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Evaluation of Molecular Technique and Microscopical Examination for Diagnosis of Blood Parasites In Equine and Camels

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ABSTRACT

This study was designed to evaluate PCR technique as a diagnostic tool for blood parasites infection in equine and camels in relation to conventional microscopic examination of stained blood smear. 150 equine stained blood smears were examined microscopically, 15 of them were suspected to be infested with equine piroplasmosis. 10 samples (including one positive by blood film) were examined by conventional PCR technique, 7 samples were positive. 135 camels stained blood smears were examined microscopically, 2 of them were infested with trypanosoma, and 10, 5 and 15 samples were suspected to be infested with theileria spp., babesia spp. and anaplasma spp., respectively. 10 samples (including 2 positive for trypanosoma), 10 negative for theileria, 10 negative for babesia and 10 negative for anaplasma by blood film were examined separately by conventional PCR technique, 9, 1, 1 and 5 samples were positive, respectively. The results concluded that PCR assays are more sensitive than blood film examination.

1. INTRODUCTION:

Equine piroplasmosis affects the development of equine industries worldwide, including Egypt, especially in the acute phase (Farah et al., 2002). Equine piroplasmosis is a disease caused by two species of intra-erythrocytic protozoa, namely Babesia caballi and Theileria equi that affects horses, mules, donkeys and zebras. Both parasites are transmitted by ticks of genera Dermacentor, Hyalomma, and Rhipicephalus (Scoles and Ueti, 2015). Equine piroplasmosis is endemic in tropical and temperate areas and occurs in acute, sub-acute and chronic forms. Typical clinical signs of EP are fever, depression, anaemia, icterus, edema, anorexia and, occasionally, mucosal petechiae and ecchymoses (De Waal, 1992).

Trypanosomiasis is the most important and serious pathogenic protozoal disease of camel caused by Typanosoma evansi, infecting a wide range of animals throughout tropical and sub-tropical regions of the world (Abdel-Rady, 2008; Barghash et al., 2014). Trypanosomiasis may be acute with high fever, weakness, anemia, high morbidity and high mortality (Enwezor and Sackey, 2005). Theileriosis is one of the most common tick-borne diseases, Theileria spp. that has been reported in camels includes T. camelensis and T. dromedarii; (Hekmatimoghaddam et al., 2012). The prominent clinical signs of camel theileriosis are fever, ocular watery discharge, severe emaciation, diarrhea in the form of intermittent bouts, in addition to the systemic signs, enlargement of superficial lymph nodes were also noticed (El-Fayoumy et al., 2005; Hamed et al., 2011). On the other hand, camels may be apparently healthy in spite of theilerial infection (Boid et al., 1985). Babesiosis in camels were manifested by Fever, in appetence, progressive anemia and hemoglobinuria, pale and or icteric mucous membranes, digestive disturbances, emaciation and sometimes sudden death (Qablan et al., 2012). Dromedary camels infected with Anaplasma marginale show fever, anorexia, diarrhea, emaciation, pale mucous membranes, lacrimation, abortion and/or infertility (Ismael et al., 2016).

Diagnosis of blood parasites in equine and camels on clinical basis is unreliable and is therefore performed using laboratory methods represented by stained blood smears, serological tests and PCR methods (Sumbria et al., 2015; Kocan et al., 2010; Li et al., 2015). The examination of the stained blood smears was simple but insufficient for accurate detection and identification of the parasite during mixed infections and in particular in carrier cases or sub-clinical infections with low parasitemia (Krause, 2003; Quintão-Silva and Ribeiro, 2003). Serological tests used for the parasite detection have disadvantages in reproducibility due to the antigenic variation and significant levels of false negative and false positive results. In addition, these tests do not distinguish between current infections and residual antibodies from previous vaccination or infection (Chansiri and Khuchareontaworn, 2002). So, the PCR has been applied with high sensitivity and specificity compared to other diagnostic methods (Mahmmod et al., 2010; Qablan et al., 2013).

The present study was undertaken to determine the reliability of PCR as compared with the microscopic examination, i.e. Giemsa stained smear method, for the diagnosis of blood parasites in equine and camels.

2. Materials and methods:

2.1. Animals:

Animal	location	Number
Horses	Alexandria { El-Gyad club-individual raised cases}	43
	Behera (individual raised cases)	50
	Total	93
Donkeys	Alexandaria zoo	15
	Behera (individual raised cases)	38
	Total	53
Mules	Behera (individual raised cases)	4
Total equine		150
Camels	Kom-Hamada-Behera abattoir	135

2.2. Sampling:

Whole blood samples were collected from all animals by jugular vein puncture using Vacutainer tubes® with anticoagulant (EDTA). Blood with EDTA were used for preparation of blood smears directly after collection, and the rest stored at -20 ◦C until used for DNA extractions for PCR assay.

2.3. Blood film preparation:

The blood smears were prepared from blood collected from animals according to the method described by **(Soulsby, 1982)**.

2.4. Molecular diagnosis:

2.4.1. DNA extraction:

Genomic DNA was extracted from the collected blood samples according to the method described by G-spin™ Total DNA Extraction Kit (INTRON biotechnology, Korya). A final DNA product of 50 µl was eluted. Following extraction, DNA samples were stored at -20 ºC until analyzed by PCR.

2.4.2. PCR reaction:

Equine: 10 blood samples (including one positive by blood film).

Camels: 10 blood samples (including 2 positive for trypanosoma), 10 negative for theileria, 10 negative for babesia and 10 negative for anaplasma by blood film.

These samples were examined by conventional PCR technique. The primers used in this study were commercially prepared and are presented in table (1). The PCR reactions were performed as shown in table (2)

Table (1): Primer Sequence used in PCR amplifications for detection of blood parasites in equine and camels:

Parasite	Primer Sequence (5' - 3')	Product size (pb)
18S rRNA gene of *B. caballi* and *T. equi*, respectively **(Allsopp et al., 1994; Criado-Fornelio et al., 2003)**	F- TCGAAGACGATCAGATACCGTCG- and R- TGCCTTAAACTTCCTTGCGAT-	392 T. equi
	F- TCGAAGACGATCAGATACCGTCG- and R- CTCGTTCATGATTTAGAATTGCT-	540 B. caballi
T.evansi	TBR1.2 F-GAATATTAAACAATGCGCAG- and TBR1.2 R-CCATTTATTAGCTTTGTTGC-	164
Theileria spp.	F-AGT TTC TGA CCT ATC AG- and R-TTG CCT TAA ACT TCC TTG-	1098
Babesia spp.	F-GTTGATCCTGCCAGTAGTCA- and R-CGGTATCTGATCGTCTTCGA-	913
Anaplasma marginale	F-GCT CTA GCA GGT TAT GCG TC- and R-CTG CTT GGG AGA ATG CAC CT-	265

Table (2): PCR conditions of different blood parasites:

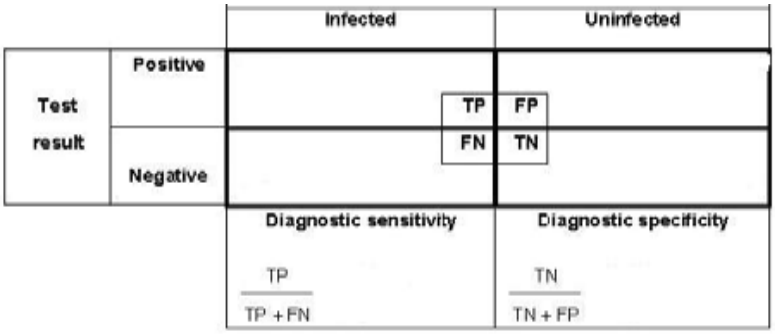
Parasite	*PCR conditions*	*References*
T. equi B. caballi	Initial denaturation at 96°C for 10 min., 40 cycles of the following conditions were repeated: denaturation at 96°C for 1 min., annealing at 60.5°C for I min., extension at 72°C for 1 min. and final extension at 72°C for 10 min.	(Alhassan et al., 2005)
T.evansi	94◦C for 10 min., 40 cycles at 94◦C for 30 sec., 50◦C for 45 sec., 72◦C for 60 sec. Post-extension was by one cycle at 72◦C for 10 min	(Masiga et al., 1992)
Theileria spp.	95◦C for 5 min., 39 cycles at 90◦C for 1 min., 52◦C for common primer, 72◦C for 1 min. and final extension at 72◦C for 10 min.	(Youssef et al., 2015)
Babesia spp.	96°C for 10 min., 40 cycles at 96°C for 1 min., 60.5°C for I min., 72°C for 1 min. and final extension at 72°C for 10 min.	(Alhassan et al., 2005)
Anaplasma marginale	95°C for 3 min., 35 cycles at 94°C for 30 sec., 57°C for 30 sec., 72°C for 30 sec., and final extension at 72°C for 7 min.	(Barghash et al., 2016)

2.5. Gel electrophoresis:

The amplified DNA samples were electrophoresed on 1.5% agarose gel and stained with ethidium bromide to visualize the amplified DNA fragments under ultraviolet light as shown in (Figure 3, 4, 5, 6 and 7).

2.6. For evaluation of PCR and microscopical blood smear examination (gold standard test), the following formulae were used to detect the epidemiological items including, specificity sensitivity and accuracy of the diagnostic test.

$$Accuracy = \frac{TP + TN}{TP + FP + FN + TN}$$



3. RESULTS

3.1. Blood film examination:

Equine: 150 stained blood smears were examined microscopically, 15 of them were suspected to be infested with equine piroplasmosis (figure 1).

Camels: 135 stained blood samples were examined microscopically, 2 of them were infested with trypanosoma (figure 2), 10, 5 and 15 were suspected to be infested with theileria spp., babesia spp. and anaplasma spp., respectively.

3.2. PCR assay:

Equine: The PCR revealed that 7 samples found positive for T. equi out of 10 examined samples (one positive and 9 negative by blood film) as shown in table (3).

Camels: The PCR revealed that 9, 1, 1 and 5 samples found positive for T. evansi, theileria spp., babesia spp. and anaplasma marginale, respectively out of the examined samples as shown in table (4).

Table (3): Incidence of equine piroplasmosis by blood film and PCR:

Animals	Blood film		PCR	
	No=150	%	No=10	%
Horses	9	6	4	40
Donkeys	5	3.3	3	30
Mules	1	0.6	0	0
Total	15	10	7	70

Table (4): Prevalence of blood parasites in camels by blood film and PCR:

Parasites	Blood film		PCR	
	No=135	%	No=10	%
T. evansi	2	1.4	9	90
Theileria spp.	10	7.4	1	10
Babesia spp.	5	3.7	1	10
Anaplasma marginale	15	11.1	5	50

- **Table (5): Sensitivity, specificity and accuracy of PCR in comparison to blood film:**

Blood smear / PCR	Positive	Negative	Total
Positive	1	6	7
Negative	0	3	3
Total	1	9	10

Equine piroplasmosis
Sensitivity of PCR= 100%
Specificity of PCR = 33.3%
Accuracy = 40%

Blood smear / PCR	Positive	Negative	Total
Positive	2	7	9
Negative	0	1	1
Total	2	8	10

Camel trypanosomiasis
Sensitivity of PCR= 100%
Specificity of PCR = 12.5%
Accuracy = 30%

- **Table (6): Sensitivity, specificity and accuracy of blood film in comparison to PCR:**

PCR / Blood smear	Positive	Negative	Total
Positive	1	0	1
Negative	6	3	9
Total	7	3	10

Equine piroplasmosis
Sensitivity of PCR= 70%
Specificity of PCR = 100%
Accuracy = 40%

PCR / Blood smear	Positive	Negative	Total
Positive	2	0	2
Negative	7	1	8
Total	9	1	10

Camel trypanosomiasis
Sensitivity of PCR= 22.2%
Specificity of PCR = 100%
Accuracy = 30%

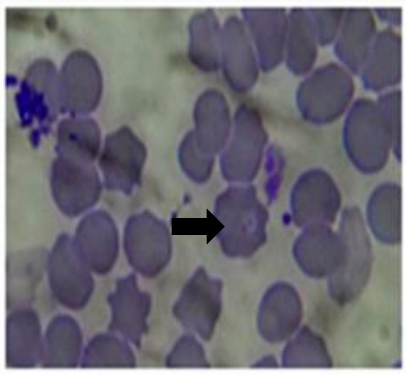
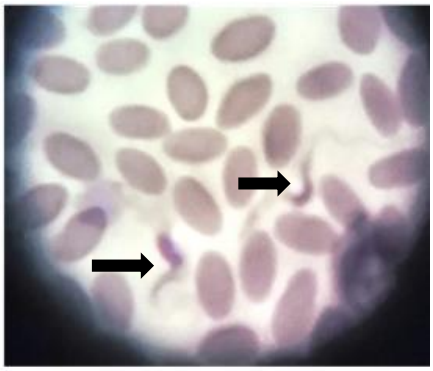


figure (1): Equine piroplasmosis Giemsa stain, x100



fiugure (2): Trypanosoma, Giemsa stain, x100

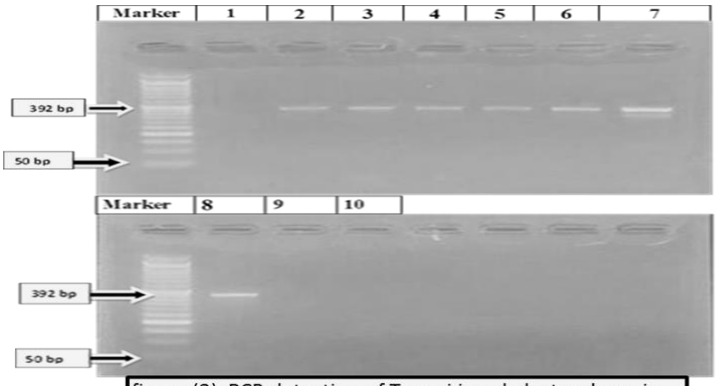


figure (3): PCR detection of T. equi in gel electrophoresis

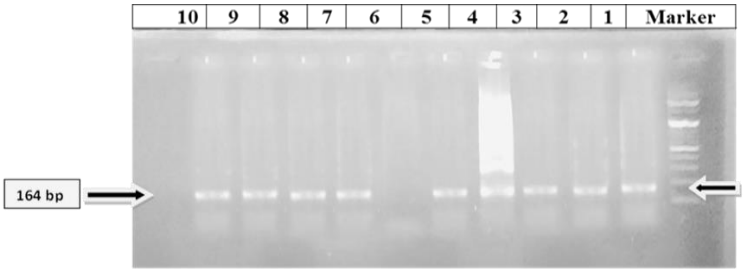


Figure (4): PCR detection of Trypanosoma evansi in gel electrophoresis

Marker (100 bp)
Lane (1, 2, 3, 4, 5, 7, 8, 9 and10): positive at 164 bp
Lane 6: negative

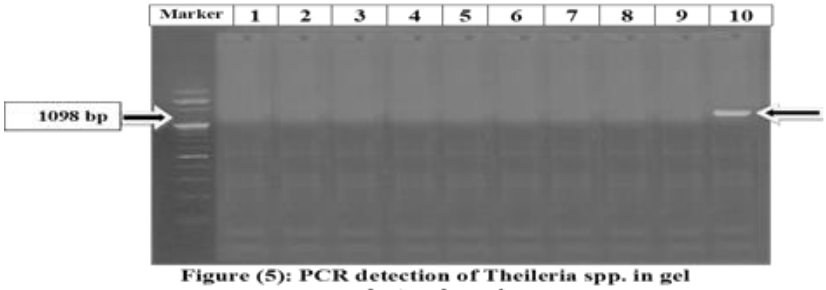


Figure (5): PCR detection of Theileria spp. in gel electrophoresis

Marker (100 bp)
Lane (10): positive at 1098 bp

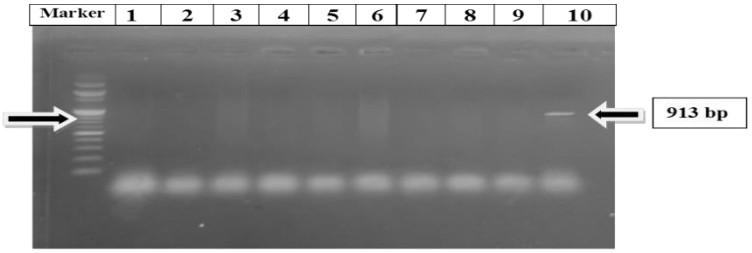


Figure (6): PCR detection of Babesia spp. in gel electrophoresis

Marker (100 bp)
Lane (10): positive at 913 bp

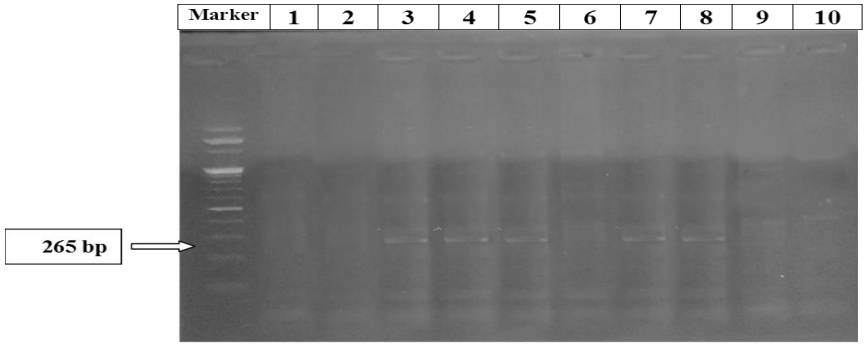


Figure (7): PCR detection of Anaplasma marginale in gel electrophoresis

Marker (100 bp)
Lane (3, 4, 5, 7 and 8): positive at 265 bp

4. DISCUSSION:

Diagnosis of blood parasites in equine and camels on clinical basis is unreliable and is therefore performed using laboratory methods represented by stained blood smears, serological tests and PCR methods (Sumbria et al., 2015; Kocan et al., 2010; Li et al., 2015).

In the current study, table (3) showed that the incidence rate of equine piroplasmosis was 10% (15 out of 150 examined animals). Our results were relatively similar to that reported by (Mahmoud et al., 2016) who found that the incidence rate in equine population was 13.6% (19 positive cases out of 139 samples) by blood film.

Detection of equine piroplasmosis by PCR assay was found to be 26% (26 out of 100) while by Giemsa staining method was 18 % (18 out of 100) in a study conducted by (Ibrahim et al., 2011). (Mahdy et al., 2016) also reported that the number of positive cases of theileriosis out of 301 equine blood samples by PCR and smear method were 171 (56.8%) and 79 (26.2%), respectively. The results obtained in our study; 70% (7 out of 10) and 10% (15 out of 150) by PCR and staining method, respectively indicate that PCR is more efficient in detecting equine piroplasmosis than the conventional staining technique and are in agreement with previous studies (Ibrahim et al., 2011; Mahdy et al., 2016; Mahmoud et al., 2016).

In our study, the Giemsa stained blood smears had shown false negative in visual examination under light microscope, which shows low sensitivity of this test (70%) as compared to PCR (100%). It may be due to several reasons like the visual mistakes made during the examination of slides, very low parasitaemia, destruction of piroplasmic forms in red blood cells due to hemolysis, the thickness, dirtiness or unsuitable blood smear staining. Moreover, the microscopic detection of piroplasms in samples that were negative by PCR tests was not possible. This fact, confirms the superiority of PCR over blood smear examination. Considering blood smear examination as the gold standard assay, the sensitivity of PCR method was found to be 100 %. Our findings are in agreement with (Ibrahim et al., 2011) who reported that sensitivity and accuracy of PCR in detection of B. equi was superior to the blood smear examination.

The difficulties found in detection and differentiation of the parasite by blood smear overcome by the molecular methods like PCR. The high efficacy and sensitivity of PCR makes it an attractive tool in diagnosing the tick-borne infections which is in accordance with (Bashiruddin et al., 1999; Friedhoff and Soule, 1996). Hence, our study clearly proves that PCR can be used for accurate diagnosis of equine piroplasmosis, and can also be used to detect the carrier animals, which serve as a potential source of infection.

In the current study, table (4) showed that the prevalence rates of trypanosoma spp., theileria spp., babesia spp. and anaplasma spp. were 1.4%, 7.4%, 3.7% and 11.1% (2, 10, 5 and 15 out of 135 examined camels), respectively. Our results were lower than those detected by (El-Naga and Barghash, 2016) who found that the prevalence rates were 20.2%, 50.8%, 11.8% and 47.4% (67, 168, 39 and 157 positive cases out of 331 samples), respectively by the staining method.

Detection of camel trypanosomiasis by PCR assay was found to be 74.7% (186 out of 249) while by Giemsa staining method was 20.9 % (52 out of 249) in a study conducted by (Barghash et al., 2014). (Abd-El-Hameed, 2016) also reported that the number of positive cases of trypanosomiasis out of 396 camel blood samples by blood smear was 23% (5.8%) and by PCR assay was 65 out of 150 (43.3%). Our results; 90% (9 out of 10) and 1.4% (2 out of 135) by PCR and staining method indicate that PCR is more efficient in detecting camel trypanosomiasis than the conventional staining technique and are in agreement with previous studies (Abd-El-Hameed, 2016; Barghash et al., 2014; El-Naga and Barghash, 2016).

Microscopic detection of blood parasites requires high parasitemia, good smear preparation, proper staining and a well-trained microscopist (in spite of the fact that the technique is cheaper and easier to perform) Furthermore, the parasites like structures recognized in erythrocytes are often difficult to differentiate from Heinz bodies, Howell-Jolly bodies or staining artifacts (Ge et al., 1995; Noaman and Shayan, 2010).

The results detected by PCR-based assay were higher than that obtained by blood film examination and this may attributed to higher sensitivity of molecular technique (Clausen et al., 1998; Penchenier et al., 1996). PCR was the powerful method used, not only when products were not detected in DNA free samples but also describe what trypanosoma, anaplasma, theileria and babesia subspecies present. Moreover, it allowed the accurate diagnosis of mixed infections which could not be detected by GSBS (El-Naga and Barghash, 2016).

The comparison of sensitivity, specificity and accuracy of the two diagnostic methods was illustrated in Table (5 , 6). PCR was found to be highly sensitive than blood film examination with a conclusion that molecular technique should be implemented of for diagnosis of blood parasites in equine and camels to overcome low sensitivity recorded by blood film.

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