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Comparative Molecular and Conventional Detection Methods of *Babesia equi* (*B. Equi*) in Egyptian Equine

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Abstract: *Theileria equi* is a tick-borne hemoprotozoan parasite and one of the causative agents of equine piroplasmosis. Blood is collected from a total of 100 horses. Three diagnostic methods: microscopical examination, enzyme linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR), were compared in detecting *T. equi*. The results showed that the prevalence rate of *T. equi* in examined horses was 18%, 30% and 26% by using microscopic examination, ELISA and PCR, respectively.Clinical, hematological and biochemical studies were evaluated in examined horses. Significant decrease in hematological parameters was observed along with an increase in some biochemical parameters. The results of PCR assay showed its importance in the diagnosis of the carrier infected cases more than with the conventional techniques. The microscopic examination was used only in acute cases of the disease.

Key words: Equine piroplasmosis · Theileria equi · ELISA and PCR.

INTRODUCTION

Equine piroplasmosis is a tick-borne disease of equine that is caused by two species of apicomplexan protozoa, *Babesia caballi* and *Theileria equi* (*Babesia equi*) [1]. The disease occurred throughout the tropical and subtropical areas of the world, with endemic areas in many parts of Europe, Asia, Arabia, Africa, South and Central America. *B. caballi* and *T. equi* were transmitted by species of ixodid ticks of the genera *Dermacentor*, *Rhipicephalus* and *Hyalomma* spp. [2]. *B. equi* was considered more pathogenic than *B. caballi* that caused a more persistent fever and anemia [3].

The clinical signs of piroplasmosis were variable and often nonspecific [4]. *T. equi* infection was generally characterized by fever, anemia, icterus and hemoglobinuria [5]. Equine babesiosis was recognized as a serious problem of major economic importance as the affected animals manifested decreased working capacity and loss of appetite so it has posed a threat to the international movement of horses [6].

Babesiosis could be diagnosed by a number of different methods, including Giemsa-stained blood smears, enzyme linked immunosorbent assays (ELISAs) and polymerase chain reaction (PCR) [7]. The examination of the stained blood smears was simple but insufficient for accurate detection and identification of *B. caballi* and *T. equi* during mixed infections and in particular in carrier cases or sub-clinical infections with low parasitemia [8, 9].

The aim of the present work was to (1) study the prevalence of *T. equi* in the collected blood samples from different Governorates of Egypt, to (2) evaluate the sensitivity, specificity and accuracy of three different diagnostic techniques [microscopy, ELISA and PCR] in the diagnosis of *T. equi* infection in examined horses and to (3) investigate the effect of *T. equi* on hematology and serum biochemistry of infected horses.

MATERIALS AND METHODS

A total number of 100 horses aged from 2 to 5 years old were clinically and laboratory examined for the presence of *T. equi* infection. The blood samples were collected by the jugular vein puncture of examined horses. Blood samples were placed in two 10-ml tubes; one tube contained di-sodium EDTA as anticoagulant, this part of blood is used for hematological analysis and DNA extraction for PCR assay, in the second one the sera were separated and stored at -20°C until used for ELISA and biochemical analysis.

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Hematological tests were performed according to Feldman *et al*. [10], including, the estimation of hemoglobin concentration, packed cell volume (PCV%), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), total white differential count, total leucocytic count (TLC) and red blood cell (RBCs) count. Three thin blood smears per animal were prepared from ear vein and stained with fields' stain for microscopic examination of parasites in red blood cells.

Serum samples were tested spectrophotometrically to determine the following parameters; alanin aminotransferase (ALT), aspartate aminotransferase (AST) activities according to Reitman and Frankel [11] and alkaline phosphatase (ALP) according to Roy [12]. Bilirubin (total, direct and indirect) was detected according to Doumas *et al*. [13]. Glucose was estimated according to Trinder [14]. Total proteins were determined according to Weichselbaum [15] and Gornal *et al*. [16]. Protein electrophoresis was performed according to Keyser and Watkins [17]. Total cholesterol was measured according to Allain [18]. Blood urea nitrogen (BUN) was measured according to Tabacco *et al*. [19]. Creatinine was measured according to Fabiny and Ertingshausen [20]. Albumin was measured according to Doumas *et al*. [21]. Calcium and phosphorus ions were measured according to the methods described by Biggs and Moorhead [22] and Goodwin [23], respectively. Sera were tested for the presence of antibodies against *T. equi* by indirect ELISA according to Voller *et al.* [24].

Genomic DNA was extracted from collected blood samples according to the method described by Qiagen genomic DNA extraction kit (Qiagen, Germany). The nucleotide sequences of the primers used in this study were shown in Table (1). The primers were synthesized by Metabion Company, Germany. The 18S rRNA gene sequences of *T. equi* [25, 26] were used for designing suitable diagnostic primers. The accession numbers used in their study were Z15105, AY150062 and AY150063 for *T. equi*. A universal screening primer pair common for *B. caballi* and *T. equi*, Bec-UF1 and Bec-UR, was designed to amplify the DNA of both parasites in one reaction. Additionally, a set of primer combinations including Bec-UF2 as a universal forward primer and Equi-R as reverse primer specific for *T. equi* was also designed for the species-specific detection.

Bec-UF1 and Bec-UF2: Universal forward primers; **Bec-UR**: Universal reverse primer, **Equi-R**: *B. equi*-specific reverse primer.

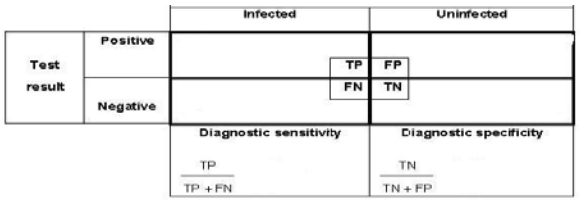
DNA amplification was performed according to the method described by Battsetseg *et al*. [27]. PCR was performed in a final volume of 50 µl containing (10 mM Tris–HCl [pH 8.3], 50 mM KCl, and 1.5 mM $MgCl_2$), 3 µl of the template DNA, 25 pmol of each of the primers used for each assay where primer pair Bec-UF1 and Bec-UR used for screening the presence of *Babesia* spp. and considered as a general primer while Bec-UF2 and Equi-R specifically amplify the DNA of *T. equi*. 20.2 mM dNTP mixture, and 2.5U of AmpliTaq Gold DNA polymerase were also added. The mixture was heated for 10 min at 96°C to activate the AmpliTaq Gold DNA polymerase, and 40 cycles of the following conditions were repeated: denaturation for 1 min at 96°C, annealing for 1 min at 55°C and 58°C for common and specific amplification, extension for 1 min at 72°C and a final extension for 10 min at 72 °C.

After layering with 40 µl of mineral oil, thermal cycling in a programmable thermal cycler was done. Positive and negative control PCR reactions were also included in this assay. Amplified DNA samples were electrophoresed on 1.5% agarose gel and stained with ethidium bromide to visualize the amplified DNA fragments under ultraviolet transilluminator. Statistical analysis of the obtained data was carried out by using one way analysis of variance (ANOVA) followed by post hoc test using the statistical package for social sciences (SPSS) computer program. All the significant differences were determined at the $p < 0.05$ level.

Table 1: Oligonucleotides primers used in PCR for amplification of 18S rRNA gene sequence of *Babesia* spp. in horses.

Primer Name	Nucleotide sequence	Annealing temperature (C°)	Product size (bp)	Species specific
Bec-UF1	5\-GTTGATCCTGCCAGTAGTCA-3\	55	913	*Babesia* spp. specific
Bec-UR	5\- CGGTATCTGATCGTCTTCGA-3\			
Bec-UF2	5\-CGAAGACGATCAGATACCGTCG-3\	58	392	*B. equi* specific
Equi-R	5\-TGCCTTA AACTTCCTTGCGAT-3\			

For ELISA and PCR evaluation against 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.



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

RESULTS AND DISCUSSION

The clinical investigation of examined horses was mentioned in Table (2). Anorexia (75%), loss of appetite (47%), fever (43%) and weight loss (35%) were considered the most frequent signs observed in infected horses, beside, pale mucous membranes, hemoglobinuria, jaundice, enlarged lymph nodes, edema of distal limbs and nasal discharge. The results of the clinical examination of the present study were in agreement with those reported by Camacho *et al*. [28], Ibrahim *et al.* [29], Sakha [30] and Takeet *et al*. [31].

The clinical signs observed in clinically affected horses of the present study might be attributed in light of explanation given by Forsyth *et al*. [32] and Glass *et al*. [33] who demonstrated that *T. annulata* spread through the lymphoid system and other organs rapidly, and induced production of tumor necrosis factor-α and interferon-δ. These cytokines disrupted the physiological integrity of the host. They added that the presence of parasites in the pituitary and adrenal glands could cause disturbance of the immune and endocrine systems.

Table 2: Clinical signs observed in examined horses (N=100).

Clinical signs	No. of infected horses	%
Loss of appetite	47	47
Anorexia	75	75
Weight loss	35	35
Nasal discharge	31	31
Fever (41C°)	43	43
Hemoglobinuria	12	12
Jaundice	15	15
Pale mucous membranes	21	21
Edema of distal limbs	6	6
Enlarged lymph nodes	6	6

In the present study, the mean values of total RBCs count, Hb concentration and PCV% (Table 3) showed a significant decrease in diseased animal group and suspected animal group when compared to apparent healthy animal group. The present hematological findings were in agreement with all those mentioned by Zobba *et al*. [34], Al-Saad [35] and Rashid *et al*. [36]. The reduction of erythrogram in infected horses with *T. equi* in the present work can be explained in light of several reasons; this might be due to anemia, as well as, to the destruction of RBCs by *Babesia* spp. [37]. The significant reduction in MCHC and significant increase in MCV recorded in infected and suspected animal groups of the present study suggested a macrocytic hypochromic type of anemia. This type of anemia was recorded in naturally infected foals with *B. caballi* and/or *B. equi* in Mosul [35].

Table 3: Changes in mean values ± SD of hematological parameters of diseased animal group and suspected animal group in comparison to apparent healthy animal group

Parameter	Unit	Apparent- healthy animal group N=70	Suspected animal group N= 12	Diseased animal group N= 18	LSD
RBCs count	$x10^6/\mu l$	7.78±0.31	5.42±0.38*	4.13±0.03*	0.56
Hb conc.	g%	12.83±0.12	10.80±0.26*	9.80±0.17*	0.45
PCV%	%	32.67±1.15	30.33±1.53*	27.67±0.58*	1.01
MCV	fl	40.72±1.03	57.72±1.65*	66.67±1.56*	4.95
MCHC	g%	41.31±0.07	34.03±2.40*	35.13±0.14*	3.69
TLC	$x10^3/\mu l$	11.14±0.38	12.77±0.42*	13.18±0.14*	0.57
Neutrophil count	$x10^3/\mu l$	5.26±0.10	6.30±1.78*	7.36±0.23*	0.43
Lymphocyte count	$x10^3/\mu l$	3.46±0.12	4.15±1.83*	3.97±0.13*	0.21
Monocyte count	$x10^3/\mu l$	1.44±0.05	1.27±0.06	1.35±0.00	0.35
Eosinophil count	$x10^3/\mu l$	0.55±0.02	0.100±0.26*	1.04±0.01*	0.23

*, Significant at p< 0.05; Hb conc., Hemoglobin concentration; LSD, Least significant difference; MCHC, Mean corpuscular hemoglobin concentration; MCV, Mean corpuscular volume; PCV%, Packed cell volume; RBCs, Red blood cells; SD, Standard deviation; TLC, Total leucocytic count.

Table 4: Changes in mean values ± SD of biochemical parameters of diseased animal group and suspected animal group in comparison to apparent healthy animal group

Parameter	Unit	Apparent- healthy animal group N=70	Suspected animal group N= 12	Diseased Animal group N= 18	LSD
ALT	IU/L	14.30±0.46	12.90±1.02	15.40±0.87	2.74
AST	IU/L	23.27±1.23	22.40±2.40	24.51±1.10	1.75
ALP	IU/L	57.20±4.27	58.90±3.33	61.5±5.83	5.58
Total proteins	g/dl	6.04±0.26	7.31±0.5	7.79±0.32*	1.44
Albumin	g/dl	3.05±0.09	3.65±0.44	3.37±0.42	0.66
Alpha globulins	g/dl	0.36±0.09	0.39±0.06	0.35±0.07	0.11
Beta globulins	g/dl	0.39±0.02	0.37±0.10	0.47±0.05	0.13
Gamma globulins	g/dl	2.36±0.06	2.66±0.06	3.42±0.13*	0.33
A/G ratio	----	1.02±0.12	0.99±0.23	0.76±0.13*	0.16
Total cholesterol	mg/dl	78.49±1.86	88.00±3.23*	84.95±1.86*	4.99
Triglycerids	mg/dl	96±1.02	122±7.94*	134.33±1.53*	13.15
Glucose	mg/dl	60.79±2.76	84.4±2.44*	92.64±5.78*	10.87
BUN	mg/dl	18.96±1.05	19.63±2.89	21.64±2.33	3.86
Creatinine	mg/dl	2.74±0.03	2.54±0.10	3.01±0.66	0.67
Total bilirubin	mg/dl	3.24±0.07	3.79±0.03*	5.86±0.05*	0.51
Direct bilirubin	mg/dl	1.29±0.04	1.41±0.05	1.49±0.07	0.31
Indirect bilirubin	mg/dl	1.95±0.03	2.35±0.03*	4.37±0.03*	0.35
Phosphorus	mg/dl	4.78±0.93	4.87±0.52	5.96±1.16*	0.83
Calcium	mg/dl	10.31±1.33	9.79±0.42	10.22±0.93	1.12

A/G ratio, albumin/ globulin ratio; ALP, Alkaline phosphatase; ALT, Alanine amino-tranferase; AST, Aspartate amino-transferase; BUN, Blood urea nitrogen; IU/L, Internationnal unit per liter; g/dl, gram per deciliter; mg/dl, milligram per deciliter.

Three mechanisms of hemolysis have been described in canine babesiosis to explain the pathogenesis of anemia, but the same causes could be ascribed to equine piroplasmosis [34]: mechanical mechanism by trophozoite intra-erythrocyte binary fission, immunomediated mechanism by autoantibodies directed against components of the membranes of infected and uninfected erythrocytes, and toxic mechanism by hemolytic factor produced by the parasite [38, 39].

In the present study a significant increase in TLC, neutrophils, lymphocytes and eosinophils count were observed in infected and suspected animal groups. These changes in leukogram were in agreement with the results of Ibrahim *et al*. [29] who proposed that the parasitic infestations usually induce eosinophilic response.

Ike *et al*. [40] indicated that in leukocytic findings, TLC increases remarkably with the rise of parasitized erythrocytes rate and the decrease in RBCs count. The increase in TLC may be also due to the stimulation of lymphoid system and bone marrow as immune response against the parasite or their toxins resulting in proliferation of lymphocytes [41].

In the present study non significant increase in the activities of ALT, AST and ALP (Table 4) was recorded in the diseased animal group when compared to the apparent healthy animal group. These changes were in agreement with those of Ibrahim *et al*. [29] and Zobba *et al*. [34]. The increase in liver enzymes observed in the present study may be due to that mentioned by Sandhu [42] who indicated that *T. annulata* infection caused damage to the hepatobiliary system due to hypoxia resulting from anemia and jaundice. The observed increase in serum hepatic enzymes in the current work may be also attributed to centrilobular degeneration and necrosis of hepatocytes [28].

A significant increase in total proteins was observed in diseased animal group as a result of increased value of globulin fractions, while albumin showed non significant change and albumin/globulin ratio was reduced significantly in diseased horses (Table 4). These results were in agreement with those of Ibrahim *et al*. [29]. Takeet *et al*. [31] related the increase in total proteins as a result of chronic inflammatory disorder of the liver such as cirrhosis.

A significant increase in total cholesterol and triglycerides (Table 4) was recorded in infected and suspected animal groups, in agreement with Yadav and Sharma [43] who attributed the increase in cholesterol in calves infected with *T. annulata* to the liver damage. A significant increase in glucose level was detected in infected and suspected animal groups.

Table 5: Prevalence of *T. equi* in examined horses (N=100) by using blood film examination, ELISA and PCR.

	positive		Negative	
Test	Cases No.	%	Cases No.	%
Blood film	18	18	82	82
ELISA	30	30	70	70
PCR	26	26	74	74

ELISA, Enzyme linked immunosorbent assay; PCR, Polymerase chain reaction.

This was explained by Nel *et al*. [44] who considered that in a hypermetabolic disease such as babesiosis, hyperglycemia was not a surprising finding. They also added that in critical illness, hyperglycemia was most often caused by increased glucose mobilization and stress, and can be markedly increased by increased cortisol secretion.

The increase of blood urea nitrogen (BUN) observed in infected horses with *T. equi* in the present study (Table 4) was corroborated by Takeet *et al*. [31]. Additionally, Camacho *et al*. [28] recorded higher levels of BUN and creatinine in infected horses with *Babesia* spp. than for control animal group; in addition, the increase in BUN was related to the dehydration of infected animals. Sandhu [42] reported that the elevation in BUN may be also due to kidney damage.

In the present study a significant increase in total and indirect bilirubin with non significant increase in direct bilirubin was recorded in diseased animal group and suspected animal groups when compared to apparent healthy animal group (Table 4). Hyperbilirubinemia observed in the present study agreed also with that mentioned by Ibrahim *et al*. [29]. Zobba *et al*. [34] demonstrated that hyperbilirubinemia was the most frequent biochemical finding widely described in natural infection and was the consequence of hemolytic anemia. The increase in phosphorus ions and the decrease in calcium ions in the diseased animal group of the present work agreed with Zobba *et al*. [34].

The prevalence of *T. equi* in the tested samples with the three diagnostic methods was illustrated in Table (5) and showed that the highest prevalence was recorded by ELISA then PCR and finally microscopic examination of blood smears. The examined animals (n=100) were classified into three groups; apparent healthy= animals are negative to *T. equi* by three diagnostic methods, the suspected= animals are negative with one or two methods and negative by other(s) and the diseased= animals are positive with three method

Microscopic examination of the Fields' stained blood smears with oil immersion lens revealed the presence of small intra-erythrocytic pear-shaped merozoites, spherical and/or ovoid stages of *T. equi* (Fig. 1) in 18 blood samples of examined horses with a prevalence rate of 18% while blood smear of the other 82 (82%) of examined horses appeared free from developmental stages of *T. equi*. The present results of the microscopic examination were in agreement with those of Rashid *et al*. [36].

It was found that, 30 (30%) of serum samples were positive for *T. equi* by ELISA (including the cases which were positive with blood film examination) and the other 70 (70%) sera were negative for *T. equi*. The low positive rate for *T. equi* observed in the present study using direct microscopic identification of the parasite in blood smears compared with serological examination and PCR was in agreement with Heim *et al*. [45], Acici *et al*. [46], Sevinc *et al*. [47] and Moretti *et al*. [48].

The detection of specific antibodies by various serological methods has been recognized as the method of choice for the identification of persistently infected animals, which were subject to restrictions in international commerce. Serological methods also facilitated the testing of large numbers of animals for epidemiological studies [49]. ELISA was preferred since it was less subject to operator error [50].

Evidence was provided that good crude antigenic preparations of intracellular parasites were difficult to produce, particularly due to the presence of host contaminant components, such as red blood fragments which made the standardization of immunological assays a crucial step by increasing the occurrence of non-specific reactions [51]. On the other hand, serodiagnosis may give false positive results in the treated horses and false negative results especially in carriers as the antibodies could be detected even 4 years after the parasites had been eliminated from the blood [52].

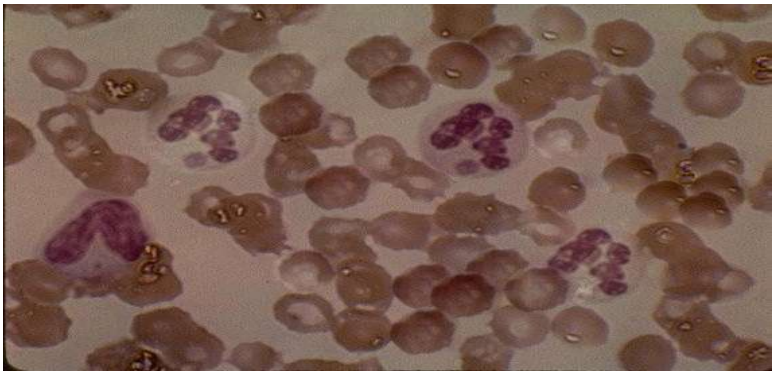


Fig. 1: Forms of *T. equi* observed in blood smears of infected horses. Fields' stain, original magnification x600).

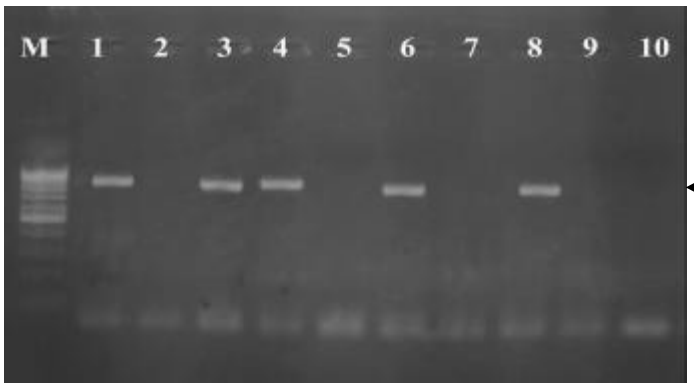


Fig. 2: Electrophoretic pattern of PCR assay for detection of *Babesia* spp. using a pair of general screening primers.; Lane M reveal 100bp ladder, lane (1) contain (+ve) DNA control samples, lane (2) contain (-ve) DNA of *Babesia* spp. while lanes 3, 4, 6 and 8 were positive samples and lanes 5, 7, 9 and 10 were negative samples.

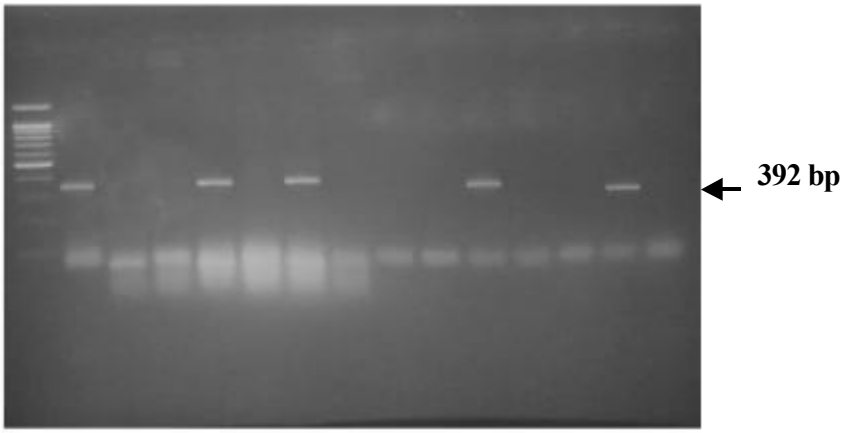


Fig. 3: Electrophoretic pattern of PCR assay for detection of *T. equi* with a pair of species specific primers.; Lane M reveal 100bp ladder, lane (1) contain (+ve) DNA control samples, lane (2) contain (-ve) DNA of *T. equi* while lanes 4, 6, 10 and 13 were positive samples while lanes 3, 5, 7, 8, 9, 11, 12 and 14 were negative samples.

DNA amplification for the diagnostic detection of equine piroplasmosis has been known as a powerful tool both in the early phase of infection and in carrier animals [53]. In the present study, a total of 26 samples (including the 18 cases which were positive with blood film examination and with ELISA), were amplified with a prevalence 26% and the target bands were detected at 913 bp by using a universal primer pair (Fig. 2) while on using species specific primers for the detection of *T. equi*, the target bands were detected at 392 bp (Fig. 3).

Table 6: Sensitivity, specificity and accuracy of both ELISA and PCR in comparison to blood film examination

Test	Sensitivity	Specificity	Accuracy
ELISA	100%	85%	88%
PCR	100%	90%	92%

These results of PCR were similar to those of Alhassan *et al*. [54] who designed a differential single-round and multiplex PCR method for the simultaneous detection of *B. caballi* and *B. equi* in blood samples collected from domestic horses in Mongolia.

The comparison of sensitivity, specificity and accuracy of the three diagnostic methods was illustrated in Table (6). PCR and ELISA were found to be highly sensitive than blood film examination while the specificity of PCR was found to be higher than ELISA with a conclusion that PCR was found to be more accurate than blood film examination and ELISA.

Bashiruddin *et al*. [4] indicated that the sensitivity of the PCR assay was higher than that of the classical microscopic examination in diagnosing piroplasmosis. PCR has been applied for the detection of many species of *Babesia* and *Theileria* and has been shown to have higher sensitivity and specificity compared with serological assays [55, 56 and 57].

In conclusion, multiplex PCR overcome the inherent disadvantage of cost and time. It simultaneously amplified multiple sequences in a single reaction that does not rely on a second round of amplification.

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