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Original Article

Application of conventional and semi-nested PCR in diagnosis of theileriosis of cattle in Egypt

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Abstract:
Theileriosis is one of the most economically important tick-borne diseases of cattle and buffaloes in tropical countries and Mediterranean region. The present study investigates 120 cows and 80 buffaloes in Cairo and Giza governorates, Egypt to evaluate PCR assay as sensitive and specific technique and microscopic examination for diagnosis of *Theileria* sp. Only 5 cows and 2 buffaloes showed clinical signs varied from rise of body temperature, enlargement of superficial lymph nodes, nasal and ocular discharge and sometimes associated with corneal opacity. The infection rate of *Theileria* in cows and buffaloes was 6 (12%) and 3 (9.3%) in Cairo and 10 (14.2%) and 6 (12.5%) in Giza respectively. The PCR result of examined blood samples of 12 cows and 8 buffaloes were positive for *Theileria* species by using 989[a]/990[a] and Tbs-S/Tbs-A primers where give 1098 pb and 430 pb respectively. None of the samples were positive for *Theileria annulata* by using N516/N517 primers. All positive samples for *Theileria* species by using Tbs-S/Tbs-A primers were used as a template and amplified by using Ta-S/Tbs-A primers specific for *Theileria annulata*, 10 out of 12 and 5 out of 8 blood samples of cow and buffaloes respectively were positive for *Theileria annulata* and give 193 bp.

Key words: Theileriosis; cattle; Egypt; PCR

INTRODUCTION

Tick-borne diseases (TBD) pose major problems for the health and management of domestic animals in tropical and subtropical regions of the world **(Jongejan and Uilenberg, 1994)**. Losses directly attributed to TBD include mortality, production losses together with the costs of veterinary diagnosis/treatment and tick as well as mortality control **(Jonsson et al., 2008)**. The most economically important tick-borne diseases is tropical theileriosis **(Georges et al., 2001)**. Tropical theileriosis caused by the protozoan parasite *Theileria annulata* of cattle and domestic buffalo (*Bubalus bubalis*). *Theileria* parasites enter the host during tick feeding as sporozoites, which rapidly invade mononuclear leukocytes. Here, they mature into macroschizonts and induce proliferation of the host cell. Macroschizonts develop further into microschizonts and ultimately into merozoites, which are released from the leukocyte. The merozoites invade erythrocytes and develop into piroplasms. Theileriosis is a lymphoproliferative disease in its early phases and is accompanied by enlargement of lymph nodes. On development of pyrexia, a lymphodestructive phase which is associated initiation of a pronounced leukopenia. Diagnosis of disease can be performed by demonstrating the forms of *Theileria* in the erythrocytes and white blood cells using Giemsa stain especially the acute phase. Since the Giemsa staining method is unspecific and can be accompanied with some technical problems and in some cases needs special experiences especially by low parasitemia.

Serological assays are more such as the immunofluorescent antibody test (IFAT), used for the diagnosis of Theileriosis suffer from the drawback that cross-reactivity of antibodies between species can obscure test specificity and pathogen identification **(Molad et al., 2006)**. Moreover, serological tests, such as the enzyme-linked immunesorbent assay (ELISA), may lack sufficient sensitivity to detect evidence of infection in samples from animal harboring low parasite burdens. Moreover, since serodiagnosis does not detect the parasite itself, whereas the animal may have already cleared the pathogen but remained seropositive. The Modern molecular techniques such as the polymerase chain reaction (PCR) allow researchers to study DNA directly. The PCR method is more accurate in comparison to serological and

microscopic methods. Using PCR method enables us to detect parasitic infections with clinical or without clinical signs.

The aim of this study was to evaluate PCR assay as sensitive and specific technique for diagnosis of *Theileria* sp. and Gold standard test (ME) from blood samples obtained from both apparently healthy (carrier) and diseased cows and buffaloes in the Giza and Cairo provinces, Egypt.

MATERIALS AND METHODS

This study was carried out on 120 cows and 80 buffaloes in Cairo and Giza governorates, Egypt.

Clinical examination:

Clinical examination was performed on all animals. The signs of *Theileria* infection were observed and recorded.

Collection of samples:

Blood samples were collected from jugular vein in tubes containing EDTA as anticoagulant for blood smear and PCR analysis. Each sample was collected and labeled separately.

Microscopic Examination:

Thin blood smears were prepared according to **Kelly (1979)** fixed by methanol and stained with Giemsa stain. The stained smears were examined under an oil immersion lens of microscope at a total magnification of x1000 for the presence of *Theileria* piroplasms. The parasites were identified according to the characters described by Pritchard and **Kruse (1982)**.

Samples for PCR:

The positive blood samples by microscopically examination were used for PCR. The samples from 12 cows and 8 buffaloes were selected to represent animals in two provinces.

Extraction of DNA from blood samples:

DNA of blood samples were extracted by using Thermo scientific kits (GeneJET Geno mic DNA Purification Kit #K0721, #K0722)

Oligonucleotide primers:

For conventional PCR, four set of oligonucleotide primers were synthesized and designed. 989[a]/990[a], N516/N517 **(Almeria, et al 2001)**, Tbs-A/ Tbs-S. and Tbs-A/ Ta-S **(Hoghooghi-Rad, et al., 2011)**. The sequence of primers used in this study are listed in the Table (1). The 989[a]/990[a] primers were constructed to target a 1098 pb specific for *Theileria* sp., while N516/N517 give 721 pb amplified product specific for *Theileria annulata*.

The Tbs-S/Tbs-A primer set was used for PCR amplification of 18SrRNA of *Theileria* sp. and the amplified sequence weight by this primer set for *Theileria* sp. was 426-430 bp. The amplified product of all PCR positive samples for *Theileria* species by using Tbs-S/Tbs-A primers were used as a template and amplified by using Ta-S/Tbs-A primers. The Ta-S/Tbs-A primer set derived from the 18SrRNA encoding gene specific for *Theileria annulata*. The amplified sequence weight by this specific primer set was 193 bp.

DNA amplification in conventional PCR

DNA amplification was done in 25 µl reaction volume containing 5 µl of Taq master ready-to-use mixes for PCR (Jena Bioscience, Cat No. 102S), 10 PM of each oligonucleotide primers, 5 µl of DNA template and fill up to 25 µl with DNAse and RNAse free water. The optimized cycle program for PCR using 989[a]/990[a] and N516 /N517 primers were as follow: initial denaturation of 5 min at 94° C; 35 cycles of 1 min. at 94°C, 1 minute at 55°C and 1 min. at 72°C; and final extension step at 72°C for 5 min **(Almeria et al., 2001)**.

Table 1: Oligonucleotide primer sequences specific for *Theileria sp*. and *Theileria annulata*

Theileria sp. primer	Sequences	Amplified product
989 [a]	5'-AGT TTC TGA CCT ATC AG-3'	1098 bp
990[a]	5'TTG CCT TAA ACT TCC TTG-3'	
N516a	5'-GTAACCTTTAAAAACGT-3'	721 bp
N517a	5'-GTTACGAACATGGGTTT-3'	
Tbs-A	*5*-'CTA AGA ATT TCA CCT CTG ACA G-3'	
Tbs-S	5'-CAC AGG GAG GTA GTG ACA AG-3'	430 pb
Ta-S	*5*-ACG GAG TTT CTT TGT CTG-3'	193 bp

The optimized cycle program for PCR using primers Tbs-S/ Tbs-A was as follow: initial denaturation of 5 min at 94°C; to denature double strand DNA, Two cycle of 94°C for 45 s (denaturing step), 55 °C for 90 s (annealing step) and 45 s in 72°C (extension step) followed by two cycles of 94°C for 45 s, 55 °C for 60s and 45 s at 72°C. This step was followed by 34 cycles of 45 s at 94°C, 45 s at 55°C and 45 s at 72°C. Finally, PCR was completed with the additional extension step at 72°C for 5 min **(Hoghooghi et al., 2011)**.

In order to show that the Tbs-S/Tbs-A PCR product was *Theileria annulata,* it was amplified in 25 µl reaction volume containing 5 µl of Taq master ready-to-use mixes for PCR (Jena Bioscience, Cat No. 102S), 10 PM of each oligonucleotide primers Ta-S/Tbs-A primer, 5 µl of the first PCR amplicon as template and fill up to 25 µl with DNAse and RNAse free water.

The optimized cycle program for the second PCR was 5 min at 95 C to denature double strand DNA, Two cycle of 94°C for 45 s (denaturing step), 55 °C for 90 s (annealing step) and 45 s in 72°C (extension step) followed by35 cycles of 45 s at 94°C, 45 s at 55°C and 45 s at 72°C. Finally, semi-nested PCR was completed with the additional extension step for 5 min in 72°C. The amplified sequence weight by this specific primer set was 193 bp **(Hoghooghi, et al., 2011)**.

Electrophoresis of PCR product:

After amplification 5 µl of the reaction product was mixed with 1 µl of 6x gel loading dye and subjected to electrophoresis on 1.5% agarose gel at 100V for 30 min.. Gel were stained with ethidium bromide and photographed on UV transilluminator. Samples were considered positive for *Theileria* species by using 989[a]/990[a] and positive for *Theileria annulata* by using N516 / 517 primers when a single band of DNA at 1098 bp and 721 bp respectively were evident in the ethidium bromide stained gels, compared with the molecular size marker (50 bp DNA ladder). Samples were considered positive for *Theileria* species by using Tbs-S/Tbs-A primers and positive for *Theileria annulata* by using Ta-S/Tbs-A when a single band of DNA at 426-430 bp and 193 bp respectively were evident in the ethidium bromide stained gels, compared with the molecular size marker (100 bp DNA ladder, Fermentas).

RESULTS

Clinical observations:

Only 5 cows and 2 buffaloes (table 2) of 120 examined cows and 80 examined buffaloes showed clinical signs varied from the rise of body temperature up to 40.5°C, the enlargement of lymph nodes specially the superficial pre-scapular lymph node, nasal and ocular discharge sometime associated with corneal opacity (Fig. 1), loss of condition and anemia in addition to the presence of ticks on different parts of the animal body. There was a slight variation in clinical signs between the cases. Others (193) were apparently healthy.

Microscopic examination

Out of 120 cows and 80 buffaloes blood samples examined by microscopic examination (Giemsa stained thin blood smears), 16 (13.3%) and 9 (11.25%) were found to be infected with *Theileria* spp. respectively. The infection rate of *Theileria* in cows and buffaloes was 6(12%) and 3 (9.3%) in Cairo and 10 (14.2%) and 6 (12.5%) in Giza respectively (table 2). The forms of *Theileria* including lymphocytic forms (Macroschizont and microschizont) (Fig. 2 A&B) and erythrocytic form with different shapes as ring, comma and oval shaped were observed in the positive microscopic samples (Fig. 3).

PCR result:

The PCR result of examined blood samples of 12 cows and 8 buffaloes were positive for *Theileria* species by using 989[a]/990[a] and Tbs-S/Tbs-A primers where give 1098 pb (Fig. 4) and 430 pb (Fig. 5) respectively. None of the samples were positive for *Theileria annulata* by usingN516/N517 primers. When the PCR product of all

Table 2: the infection rate of *Theileria* spp. by using microscopic examination of stained blood smears

Provinces	Cairo		Giza		Total	
Animals	**Cows**	**Buffaloes**	**Cows**	**Buffaloes**	**Cow**	**Buffaloes**
Total examined animals	50	32	70	48	120	80
Animals with clinical signs	2 (4%)	-	3(4.2%)	2 (4.16%)	5 (4.1%)	2 (2.5%)
Total Positive by Blood smear	6 (12%)	3 (9.3%)	10 (14.2%)	6 (12.5%)	16 (15%)	9 (11.25%)

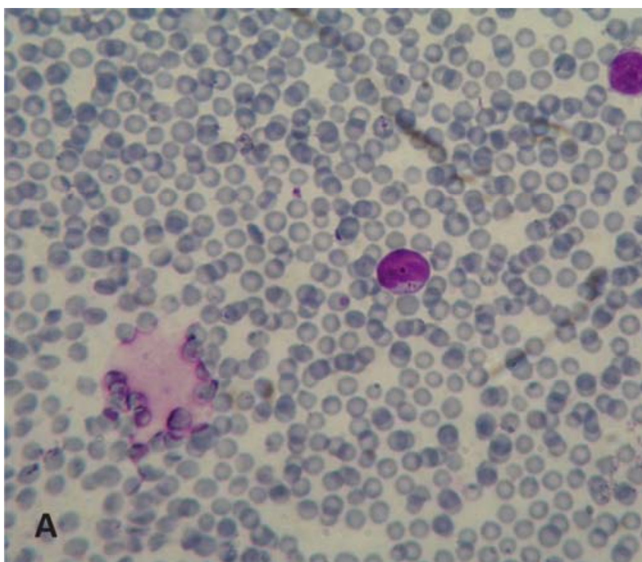
positive samples for *Theileria* species by using Tbs-S/Tbs-A primers were used as a template and amplified by using Ta-S/Tbs-A primers specific for *Theileria annulata*, 10 out of 12 and 5 out of 8 blood samples of cow and buffaloes respectively were positive for *Theileria annulata* and give 193 bp (Fig. 6).

Discussion

Seven species of *Theileria* are known to infect cow and buffaloes; of these, *T. annulata* is of major importance **(Hasanpour *et al.*, 2008)**. There is a need to develop sensitive tools for the effective detection and treatment of *Theileria* sp. in order to decrease the economic losses by the parasites. In the present study, seven animals (5 cows and 2 buffaloes) were clinically infected by *Theileria.* The observed clinical signs were fever, enlargement of superficial lymph nodes, lacrimation and corneal opacity. These findings were in agreement of **Mahmmod et al. (2011), Ali and Radwan (2011) El-Deeb and Younis (2009), Ibrahim et al. (2009), Hussein et al. (2007), Omer et al. (2002), Radostits et al. (2000), Sandhu et al. (1998), Al-Gaabary (1991)** and **Shehata et al. (1984)**.

Concerning to the microscopic examination, the obtained results from Giemsa stained blood smears revealed that 16 samples (15%) and 9 (12.5%) were positive for *Theileria* sp. in cows and buffaloes respectively. In general, the infection rate of *Theileria* in Giza was higher than in Cairo which was 14.2% in cows of Giza province corresponding to 12% in Cairo. As well as, the infection rate in buffaloes

Figure 1: Corneal opacity in cow infected with *Theileria*.



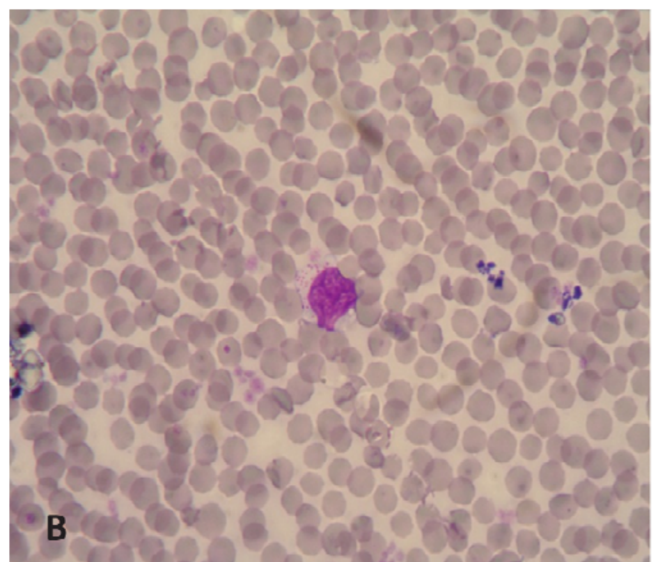


Figure 2: Blood Smear Showing macroschizont (A) and microschizont (B) in lymphocytes.

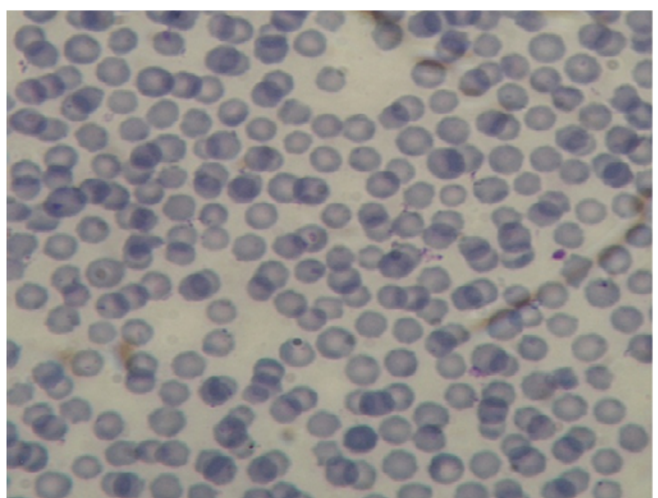


Figure 3: Blood Smear Showing ring form of *Theileria* in erythrocytes.

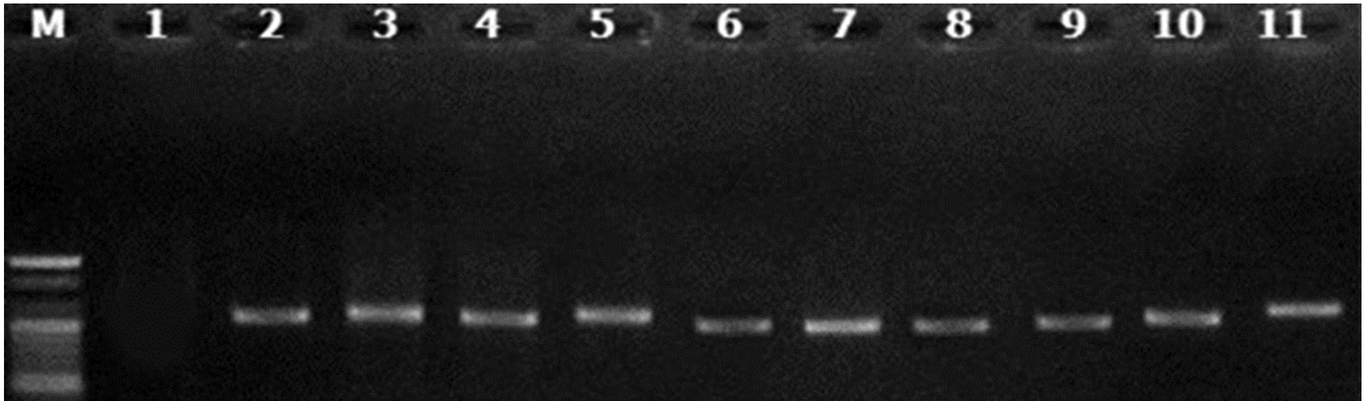


Figure 4: Ethidium bromide stained 1.5% agarose gel electrophoresis showing PCR amplified fragment of expected size 1098 bp (lane 2-11) resulted from amplification of DNA extracted from blood samples using 989[a]/990[a], a primer specific to Theileria sp. Lane M: AccuLadder 100 bp DNA size marker (Bioneer Pacific).

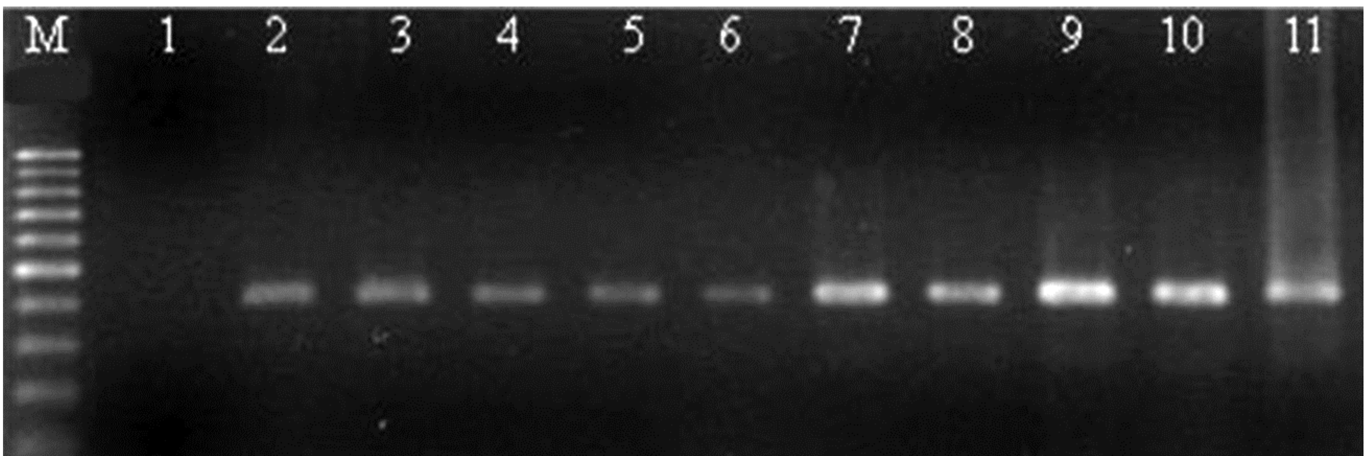


Figure 5: Ethidium bromide stained 1.5% agarose gel electrophoresis showed PCR amplified fragment of expected size 430 bp (lane 2-11) resulted from amplification of DNA extracted from blood samples using Tbs-S/Tbs-A primers specific to Thileria sp.. Lane M: 100 bp marker (fermentas).

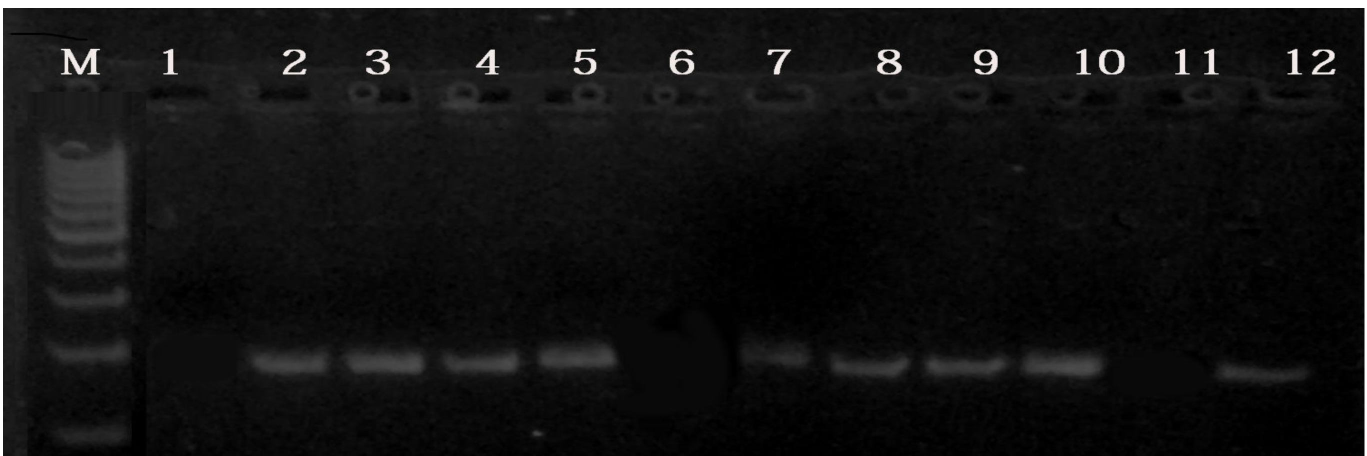


Figure 6: Ethidium bromide stained 1.5% agarose gel electrophoresis showed PCR amplified fragment of expected size 193 bp (lane 2-12) resulted from amplification of the first PCR amplicon using Ta-S /Tbs-A primers specific to *Theileria annulata*. Lane M: 100 bp marker (fermentas).

was 12.5% in Giza in corresponding to 9.3% in Cairo. These results are very close to those mentioned by **Nayel et al. (2012)** who recorded 16.05 % of examined Giemsa stained cattle (405) were infected with *Theileria sp*. in Monofia province, **Bhutto et al., (2012)**14.285% of buffaloes were infected in Pakistan and **Adel (2007)** 11.31% of buffaloes in Gharbia province. A high infection rate was recorded in cows by **Mohamed et al (2012)** and **Gamal El-Dien (1993)**. They found that the infection rate with *Theileria* sp. were 58.3% and 65.4 % respectively. In

buffaloes, a high infection rate was recorded by Ali and **Radwan (2011)** 50% (50/100) in Egypt and **Durrani et al. (2008)** 39.9% (134/336) in Pakistan while. A variable infection rates were recorded by many authors such as **Mahmmod et al. (2010)** 18.6% (48/258), **Abdou et al. (2005)** 29.4%. **Abdel Rady et al. (2010)** recorded different infection rates in different provinces ELWady EL-Geded governorate (13%). followed by Assiut governorate (12.7%), EL-fayoum governorate (8.6%), El-Minia governorate (3.8%) and finally, Sohag governorate (2.7%). Such variations may be attributed to the difference of environmental conditions that affect both parasites and vectors and immune status of the animals. The microscopic examination of blood smears revealed the forms of *Theileria* including lymphocytic (Macroschizont and microschizont) and erythrocytic forms with a ring shaped were observed in the positive microscopic samples. These results are in agreement with **Ramazan and Ugur (2006)** and **Ali and Radwan (2011)**.

In this study two set of common primers specific for Genus of Theileria species were used in PCR, 989/990 specific small subunit (SSU) rRNA primers and Tbs-S/Tbs-A derived from 18srRNA encoding gene. The two set of primers showed the same sensitivity and generated the expected DNA fragment in all *Theileria* species examined, confirming the presence of *Theileria* DNA.

By using N516/N517 primers specific for *Theileria annulata*, PCR failed to amplify target sequences from the gene encoding the 30-kDa major merozoite surface antigen. It may be these primers are not compatible with the target sequenceor introns may be present between the primer sites which, if long enough, will result in incomplete product and thus no amplification.

When the PCR product of all positive samples for *Theileria* species by using Tbs-S/Tbs-A primers were amplified by semi-nested PCR with *Theileria annulata* specific primer set (Ta-S/Tbs-A) derived from 18 srRNA encoding gene, 10 out of 12 and 5 out of 8 blood samples of cow and buffaloes respectively were positive for *Theileria annulata* and give 193 bp. Our results demonstrate that this PCR assay discriminate *T. annulata* from nonpathogenic *Theileria* species.

Several studies documented that the PCR assays aremore sensitive and specific than conventional diagnostic techniques in determining carrier animals where The PCR can detect two to three parasites per ml of infected blood, which corresponds with a parasitemia of 0.000048%. In Iran, **Azizi et al. (2008)** reported the positive infection rate of 140 carrier cattle was 40% by PCR and only 8.1% by Giemsa staining method.

Hoghooghi-Rad et al. (2011) reported that Semi-nested PCR based on detecting *Theileria annulata,* was capable of showing the cases which their Giemsa stained blood smears were false negative or false positive in visual examination under light microscope. It seems that the false negative results may be due to very low parasitaemia, destruction of piroplasmic forms in red blood cells as a consequence of deletion of typical shape of parasites in RBCs, the thickness, dirtiness or unsuitable blood smear staining.

In conclusion, the PCR especially semi-nested PCR are highly specific and sensitive methods for identifying the species of *Theileria annulata* and screening the carrier cattle in the epidemiological surveys. On the other hand, Giemsa staining method is not suitable for detecting the carrier or chronic phases of *Theileria* infection, although it is an easy and fast diagnostic technique for detecting this infection in acute phase with clinical signs in cattle.

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