IJAVMS

Using Polymerase chain reaction (PCR) for Diagnosis of Bovine Theileriosis in Upper Egypt

Ahmed Abdel-Rady, Laila S. Ahmed, Amr Mohamed[1] and Amira Al-Hosary
Department of Animal Medicine (Infectious diseases), [1]Department of Animal Medicine (Clinical Laboratory Diagnosis), Faculty of Veterinary Medicine, Assiut University, Assiut, Egypt
Corresponding author: EL_Rady@hotmail.com

Abstract

The present study was conducted on the period from April 2008 to July 2009 and included at 150 cattle and 35 Egyptian buffalo. The age of these animals ranged from one day to above five years old. The animals belonged to farms and villages of EL-Wady EL-geded, Assiut, ELFayoum, EL- Minia and Sohage Governorates.

The results of the present study cleared that the (Tams-1 primer) based PCR assay was the most sensitive test in detection of the infection with tropical theileriosis in all cases (acute, chronic and carriers). The infection rates in blood and lymph samples taken from cattle were 65.6% and 45.3%, respectively. On the other hand the infection rates were 16.7% and 25% in blood and lymph samples taken from buffaloes, respectively. PCR used as golden standard test to evaluate the conventional tests. The sensitivity of this method was 58.3% and 50% in cattle and buffaloes, respectively. While the specificity were 100% in both cattle and buffaloes.

We concluded that, Tams-1 target–based PCR is the most sensitive and specific test used for diagnosis of the disease in either acute or chronic cases and also in carrier animals of tropical theileriosis.

Keywords: Tropical Theileriosis – Cattle – Buffaloes – Diagnosis - PCR

Introduction

Tropical theileriosis (*Theileria annulata* infection, also known as Mediterranean fever). It is considered one of the tick-borne diseases that cause losses in the productivity of indigenous breeds as well as fatalities in imported foreign breeds **(13).** In Egypt, the disease is considered to be one of the most destructive obstacles to live stock production **(2, 6, 1).**

The application of genotypic assays for the diagnosis of bovine theileriosis has shown recent advances **(14).** Molecular identification provides two primary advantages to phenotypic identification; a more rapid turn around time, and an improved accuracy of identification. Polymerase chain reaction (PCR) offers important advantage such as the greater sensitivity and specificity over conventional techniques in detecting both piroplasm-infected and carrier animals. This has been verified in a number of studies performed on a wide range of animals **(11, 3, 27, 4).**

The technology of the polymerase chain reaction (PCR) is available to amplify minute quantities of parasite DNA one million– fold, thereby greatly increasing the sensitivity of DNA probes **(7, 17).**

A specific PCR was developed to test whole blood samples from *Theileria annulata* – carrier cattle. The PCR method has its superiority in separating parasitic infection associated with clinical signs (clinical form) and the infection without clinical signs (sub clinical form) **(14, 8).**

PCR–recently has been the most preferred method for detection of "Theileria" species in epidemiological studies. Several studies documented that this method is more sensitive and specific than other conventional diagnostic techniques in determining piroplasm–carrier animals **(12, 19, 15, 5).**

Using the 30 KDa major merozoite surface antigen gene (PCR) is more appropriate **(21, 9, 22).** Tams–1 gene is the most abundant and immunodomoninant antigen on the surface of merozoites and piroplasms of *Theileria annulata*. Tams–1, is a molecule with a molecular mass of approximately 30 KDa **(24, 16, 20).**

Materials and Methods

Animals:

A total number of 150 cattle (*Bos taurus*) and 35 buffalo (*Bubalus bubalis*) belong to different localities in EL-Fayoum, EL-Minia, Assuit, Sohage and EL-Wady EL-Gaded governorates were subjected to this study.

All animals showed acute or chronic forms of tropical theileriosis and other animals show no clinical symptoms but showing the different degrees of tick infestation, these were considered as carrier animals. The blood film and lymph smear were collected from 108 animal and subjected to both blood and lymph smear examination but the rest of samples were subjected to blood smear only because the enlarged lymph smear not present.

Sampling:

1. Blood sample was collected directly from the ear vein and used for preparation of blood films.
2. Whole blood sample on E.D.T.A as anticoagulant (1mg/1ml) from suspected animals by jugular vein puncture and marked with numbered labels in the field and then stored at (-20 °c) till use for DNA-extraction.
3. Lymph node aspiration were collected from enlarged lymph nodes in the sterile tube and needle after disinfections the site of introduce the needle, part of these sample used for preparation of lymph smears immediately after collection and the rest of the sample was stored at -20 ºc till used for DNA-Extraction

Clinical Examination:

All animals in this study were subjected to clinical examination, those animals showed various degrees of the characteristic clinical signs for the tropical theileriosis like fever (>40°c), enlargement of the superficial lymph

nodes (acute form), in appetence, pale or congestion of the visible mucous membranes, conjunctivitis, severe congestion of the eyes, excessive lacrimation, corneal opacity, various degrees of respiratory signs from serous nasal discharge to cough, bloody purulent nasal discharge and dyspnoea (chronic form). In addition to various degrees of ticks infestation.

Conventional diagnosis:

Thin blood films were prepared immediately after taking the whole blood samples direct from the ear vein also the lymph smears were prepared immediately in the field to allow these smears to dry by air then both samples were fixed by using methyl alcohol (methanol) for about 3-5min., allow them to dry by air after fixation step then stained with Giemsa stain diluted at 8% with bidistalied water for about 30-45 min. Dried by air and examined on Olympus microscope by using Oil immersion lens at x1000 magnification.

Molecular diagnosis:

For genetically confirmation of *T. annulata* infection, the *T. annulata* specific target (Tams-1) sequence was amplified using polymerase chain reaction (PCR).

DNA - Extraction:

DNA extraction from whole blood samples was carried out according to commercial kits **(manufacturer's instructions of QIA amp blood kit, Qiagen, Ltd, UK). As the following:**

DNA Extraction:

The samples leaved at room temperature for thawing, good mixing and then vortex. Take about 200 µl of the sample (blood or lymph) then add 20 µl of proteinase-k and vortex, add 200 µl of LA buffer (lyses buffer), incubate the samples in water bath at 56 ºc for 10 minutes, add 200 µl of absolute ethyl alcohol and vortex. Centrifugation at 10000 rpm for 1 minute in (Hettich, Zentrifugen universal 320, Germany), then transfer the samples to the spin column and centrifugation at 10000 rpm for 1 minute, then exchange the collecting tubes of the column. Add 500 µl of washing buffer 1 (AW1) and centrifugation at 10000 rpm for 1 minute, and then exchange the collecting tubes of the column, then add 500 µl of washing buffer 2 (AW2) and centrifugation at 10000 rpm for 1 minute, and then exchange the collecting tubes with micro centrifuge tubes of 1.5 ml. Add 100 µl Elution buffer and leave the tubes at room temperature for 1 minute, then centrifugation at 10000 rpm for 2 minutes, then DNeasy membrane removed and the filtrate containing the extracted and purified DNA was stored at -20 ºc till used for DNA amplification.

DNA amplification by polymerase chain reaction (PCR):

In this study the primer that used to amplifying a 785 bp fragment of the *Theileria annulata* 30 KDa major merozoite surface antigen gene. The primer sequences used were as follows **(21, 9, 22)** are deposited at GenBank™ under accession numbers AF214794–AF214920.

For the standard PCR, primer **Tams-1 F** (ATG CTG CAA ATG AGG AT) and **Tspms1 R** (GGA CTG ATG AGA AGA CGA TGA G). The final volume for DNA amplification was (25 µl) in 0.2 ml thin walled PCR tubes and this volume were containing: 12.5 µl Master mix. In liquid form (20 mM Tris – Hcl "PH 8.4", 50 mM kcl, 0.1 mM each of the four dNTPs "d ATP, d CTP, d TTP, d GTP", 1.5 mM of $MgCL_2$, 1.5 units of RED Taq DNA polymerase), 5.5 µl DNase, RNase free water, 50 pmol of the each oligonucleotede primers and 5 µl of DNA sample.

Cycling conditions:

Cycling conditions were as follows 94°c for 5 min., followed by 37 cycles consisting of 1 min at 94 °c, 1 min. at 55 °c, 2 min. at 72 °c and final extension step at 72 °c for 10 min. longer then the samples were stored at 4C° until use in the next step. The cycling condition carried out in thermocycler (**TECHNE TC – 312)**

In addition to positive control sample contain "DNA from *Theileria annulata* infected blood" and negative control sample that contain no DNA.

Gel Electrophoresis:

The chamber was connected to 75 volt power supply for 1:30 hour.10µl of each PCR product were separated by electrophoresis on 1.8 % agarose gel (GX 040.90, Gen AGarose, L.E., Standard DNA /RNA agarose, Molecular Biology Grade, Inno – Train Diagnostic, D – 61476, Kronberg / Taunus) Containing Ethidium bromide as 1 µl /ml electrophoresis buffer, using 100 bp DNA–ladder in (SCIE – PLAS , HU 10 , 5636 , UK).Then the result obtained through High performance ultraviolet transilluminator , (UVP , INC , UK) .

The image of the PCR products containing the DNA sequence of 785 bp were amplified using DOC – It ® LS, Image acquisition – soft ware, (UVP, INC, UK).

Evaluation of conventional diagnostic methods:

Evaluation of conventional methods of diagnosis against PCR test as a golden standard test was carried out in the current study. Evaluation parameters included sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and combined predictive value (CPV). The sensitivity of the test system measured the ratio of test positives to real positive. It shows how many of the real positives will be classified correctly with the test results and it calculated as (true positive / true positive + false negative). The specificity of the test system measured the ratio of test negatives to real negatives. It shows how many of the real negatives

will be classified correctly with the test results and it calculated as (true negative/ true negative +false positive). The reliability of the test results was described with its predictive values. It shows how many of the test positives and negatives are real positives and negatives, (PPV= true positive / true positive + false positive, NPV= true negative / true negative + false negative). A combined predictive value for positives and negatives can be calculated too, (CPV= true positive + true negative/ true positive + true negative + false positive + false negative) according to **(23, 26).**

Results

Clinical Examination:

The results of clinical examination reveled that the infected animals showed various degrees of the characteristic clinical signs for the tropical theileriosis like fever (>40°c), enlargement of the superficial lymph nodes, in addition to various degrees of ticks infestation (figure.1), in appetence, pale or congestion of the visible mucous membranes, conjunctivitis, severe congestion of the eyes, excessive lacrimation, corneal opacity (figure.2), various degrees of respiratory signs from serous nasal discharge to cough, bloody purulent nasal discharge and dyspnoea.

Conventional Diagnosis:

The rate of conventionally confirmed infection among clinically suspected animal were recorded based on examination of both blood and lymph smears, the results revealed that according to Giemsa stained thin blood film examination **(figure 3),** the percentages of infection were 25.3% and 8.6% in cattle and buffaloes, respectively. On the other hand, according to Giemsa stained lymph smears examination **(figure 4)**, the percentages of infection were 55.8% and 15.4% in cattle and buffaloes **(table 1).**

The relation between the finding of blood film and lymph smear examination were studied in 108 animal that were subjected to both blood and lymph smear examination. The study gave those more positive cases 48.2% were recorded with lymph smear examination as compared to 36.1% using blood film examination **(table 2).** The study revealed that out of the clinically suspected cases (13%) were conventionally confirmed positive at EL-wady EL-gaded governorate as compared to 12.4%, 8.6%, 3.8%, 2.7% were recorde at Assuit, EL- fayoum, EL-minia and Sohage Governorates, respectively**.**

Molecular diagnosis using Tams-1 target based PCR assay:

In the current study genotypic finding using Tams-1 target based PCR assay was used for molecular confirmation of *T. annulata* infection among selected cases **(figure 5)**. The obtained results confirmed the infection in 65.6% and 45.3% among selected clinically suspected cattle using blood and lymph samples, respectively **(table 3).** On the other hand, the rate of confirmed infection among selected buffaloes was 16.7% and 25% using blood and lymph samples, respectively**.**

Evaluation of the conventional methods for diagnosis of *T. annulata*:

The finding of the evaluation study of (64) examined cattle recorded a true positive, true negative, false positive and false negative results of the conventional test as 28, 16, 0 and 20, respectively. Accordingly the estimated sensitivity, specificity, PPV, NPP and CPV of the conventional diagnostic method were 58.3%, 100%, 100%, 44.4% and 68.8%, respectively (**Table 4**). The finding of the evaluation study of (12) examined buffaloes recorded a true positive, true negative, false positive and false negative results of the conventional test as 2, 8, 0 and 2, respectively.

Accordingly the estimated sensitivity, specificity, PPV, NPP and CPV of the conventional diagnostic method were 50%, 100%, 100%, 80% and 83.3%, respectively (**Table 4**).

Table 1. Rate of conventionally confirmed infection among clinically suspected cases

Animal species	Blood film			Lymph smears		
	No. of tested animals	positive	%	No. of tested animals	positive	%
Cattle	150	38	25.3	95	53	55.8
Buffaloes	35	3	8.6	13	2	15.4

Table 2. The correlation between blood film and lymph smear as conventional methods for diagnosis of tropical theileriosis

No. of examined animals (108)*	Conventionally confirmed cases			
	Blood film		Lymph smears	
	Positive	%	Positive	%
	39	36.1	52	48.2

* Total no. of animals subjected to both blood film and lymph smear examination

Table 3. Rate of genetically confirmed infection among selected clinically suspected cases using (Tams-1) target – based PCR assay

Animal species and samples	Blood		Lymph	
	Positive	%	Positive	%
Cattle (64)	42	65.6	29	45.3
Buffaloes (12)	2	16.7	3	25

Table 4. Evaluation of conventional method for diagnosis of *Theileria annulata* against PCR assay in cattle* and buffaloe**

Diagnostic methods	Test Results				Evaluation parameters (%)				
	TP a	TN b	FP c	FN d	Sensitivity	Specificity	PPV e	NPV f	CPV g
Conventional method*	28	16	---	20	58.3	100	100	44.4	68.8
Conventional method**	2	8	---	2	50	100	100	80	83.3

* PCR finding were considered as the golden standard test.
a – true positive b - true negative
c - false positive d - false negative
e - positive predictive value
f - negative predictive value
g- combined predictive value

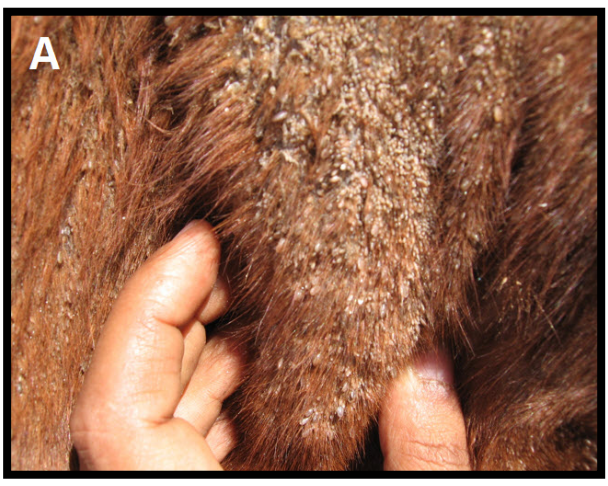

Fig.1. A. Enlargement of superficial lymph nodes.

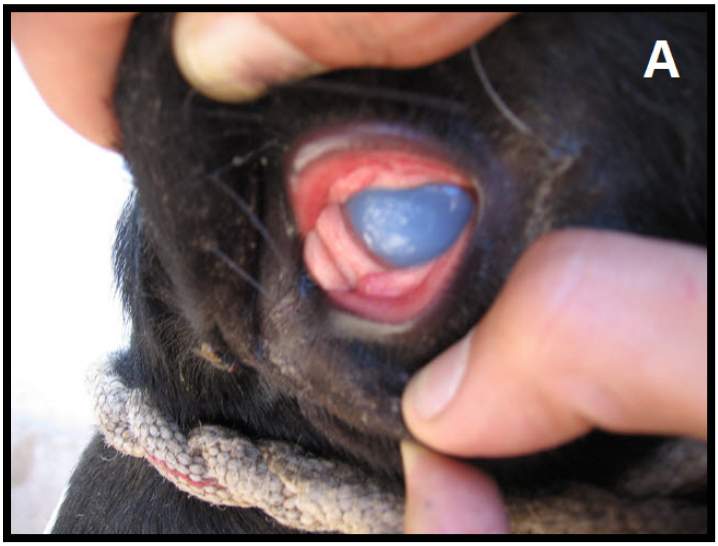

Fig.2. Ocular lesions in cows infected with tropical theileriasis A) Corneal opacity.

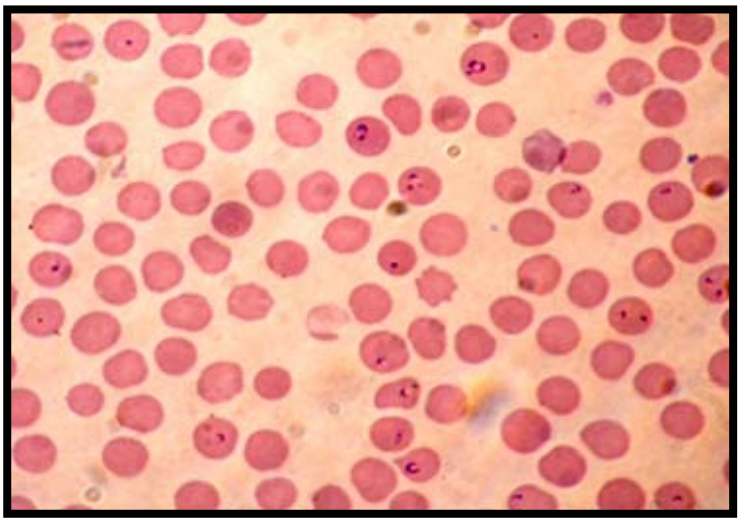

Fig.3. Blood film stained with Giemsa stain showing intracellular trophozoit "signet ring" of Theileria annulata (X100).

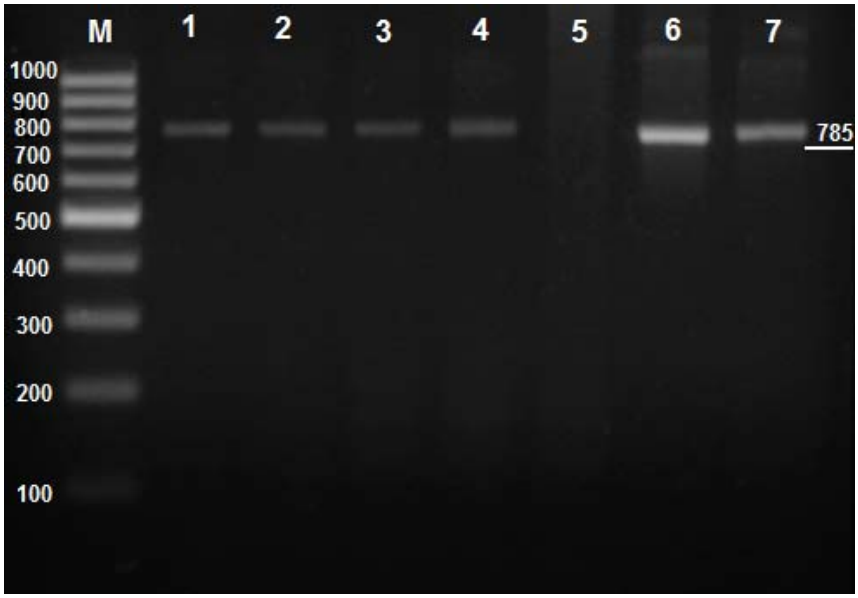

Fig. 5. Agar gel electrophoresis of PCR amplified DNA from *Theileria annulata* infested animal M, 100 bp DNA Marker DNA Lines 1-4 and 6 and 7: positive samples yielded 785 bp PCR product, Line 5: negative sample

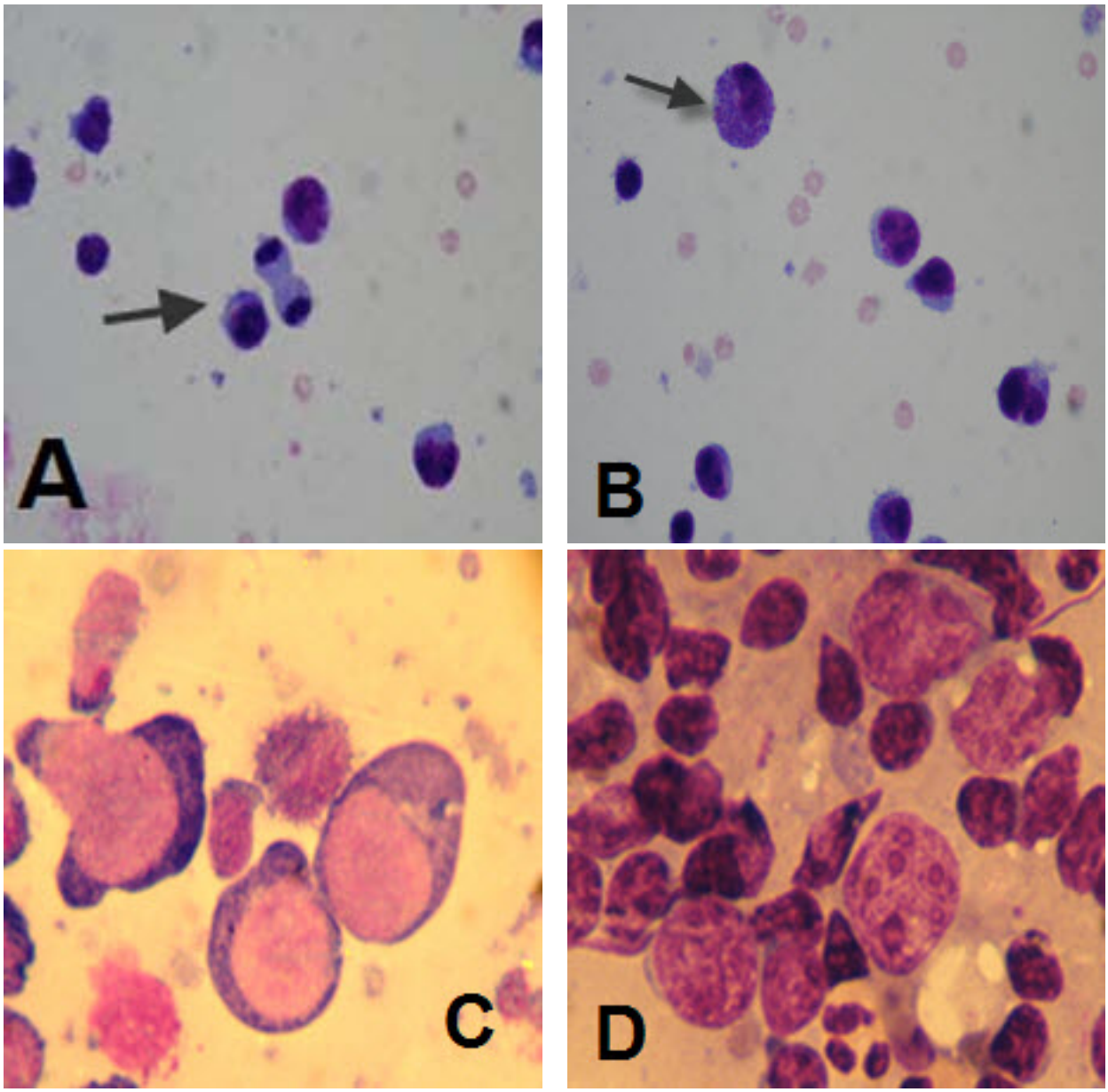

Fig.4.Lymph smears stained with Giemsa stain showing intralymphocytic schizoints of the *Theileria annulata* (X100).
A) Mitotic division of lymphocyte infected with *Theileria annulata* (Lymph proliferation).
B & C) Intralymphocytic microschizoints of the *Theileria annulata*
D) Intralymphocytic macroschizoints of the *Theileria annulata*

Discussion

In the current study *Theileria annulata* infection among clinically suspected cattle and buffaloes were studied using conventional and molecular diagnostic assays. In the current study a total number of 150 cattle and 35 buffaloes were selected based on the characteristic clinical signs of the *Theileria annulata* infection and were considered as clinically-suspected cases of tropical theileriosis. The inclusion criteria included animal with different degree of tick infestation, fever, enlargement of superficial lymph nodes, respiratory affections, and/or ocular lesions like corneal opacity and sometimes only lacrimation, with or without congestion of the visible mucous membranes. Similar clinical signs were recorded in previous studies in cattle and buffaloes, the enlargement of superficial lymph nodes and the ocular lesions in calves. In Egypt reported the same clinical signs in cattle**.** In Egypt reported similar clinical signs in buffaloes which include (fever, enlargement of superficial lymph nodes, respiratory affections, and/or ocular lesions like corneal opacity, lacrimation, paleness of the visible mucous membranes) **(6, 1)**.

In the present study we found that using of conventional methods for diagnosis of tropical theileriosis, although they are cheap and easy to use, was limited for the detection of acute cases especially with high level of

parasitemia. The test was less sensitive during the chronic form or in case of carrier animals. The obtained results for the conventional method of diagnosis were (48.4% and 16.7%) in cattle and buffaloes, respectively.
The current study revealed that examination of Giemsa stained lymph smears can provided more diagnostic value (48.2%) if compared with the Giemsa stained thin blood films (36.1%). This was manifested by the high percentage rate of positive cases which recorded from examination of lymph smears in comparison with others obtained from blood smears. This could be attributed to the easier detection of Koch's blue bodies
Inside the lymphocytes of the stained lymph smear than detection of intraerythrocytic trophozoites of the *T. annulata* in stained thin blood film. This result was in agreement with **(18)** in Germany and **(1)** in Upper Egypt they reported that it was the method of choice for diagnosis of tropical theileriasis in suspected cases specially those with enlarged lymph nodes.
Polymerase chain reaction (PCR) offers important advantage such as the greater sensitivity and specificity over conventional and serological techniques that has been verified in a number of studies performed on a wide range of parasites. Tams-1 target-based PCR assay has its superiority in separating parasitic infection associated with clinical signs (clinical form) from those without clinical signs (sub-clinical form) **(11, 14, 16, 21, 8, 3, 27, 4, 5).**
In the current study Tams-1 target based PCR assay was used for genetical confirmation of *Theileria annulata* infection using lymph and/or blood specimens. The obtained results confirmed the infection in 65.6% and 45.3% of blood and lymph specimens from selected cattle. On the other hand, the infection was confirmed in 16.7% and 25% of blood and lymph specimens from selected buffaloes. These finding clearly indicate that the rate of infection among buffaloes is low as compared with that of cattle. This could be contributed to the ability of buffaloes to resist the infection through innate factors and cell mediated immune response in addition to low affinity of the protozoan parasite (*T. annulata*) to the buffaloes' cells as previously mentioned by **(25, 10).**

References

1. Abdel–Rady, A.; Abd Ellah, M.R. and Kotb, S. (2008): Clinical, Diagnostic and therapeutic studies on theileriasis (Theileria annulata) in cattle in Upper Egypt. S. C. V. M. J., XIII (2) p.p. 387-395.

2. Ahmed, A. B. (1980): Some biological studies on blood parasites in farm animals. Ph. D. Thesis, Fac. Vet. Med. Zagazig Univ.

3. Aktas, M.; Dumanli, N.; Ce nkaya, B. and Cakmak, A. (2002): Field evaluation of PCR in detecting Theileria annulata infection in cattle in eastern Turkey. Vet. Rec., 150: 548–549.

4. Aktas, M.; Altay, K. and Dumanli, N. (2005): prevalence and distribution of tropical theileriosis in eastern Turkey. Vet. Parasitol. 127: 9–15.

5. Aktas, M.; Altay, K. and Dumanli, N. (2006): A molecular survey of bovine theileria parasites among apparently healthy cattle and with a note on the distribution of ticks in Eastern Turkey. Vet. Parasitol. 138:179 – 185.

6. Al–Gaabary, M. H. (1995): Epidemiological and immunological studies on bovine tropical theileriosis. Ph.D. Thesis. Department of Animal Medicine, Faculty of Veterinary Medicine, Zagazig university

7. Allsopp, M.T.E.P.; Theron, J.; Coetzee, M.L.; Dunsterville, M.T. and Allsopp, B.A. (1999): The occurrence of Theileria and Cowdria parasites in African buffalo (Syncerus caffer) and their associated Amblyomma hebraeum ticks. Onderstep. J. Vet. Res., 66: 245-249.

8. Almeria, S.; Gutierrez, J. F.; Estrada–Pena, A.; Ortuno, A.; Ferrer, D. and Castella, J. (2001): Bovine piroplasms in Minorca (Balearic Islands, Spain): A comparison of PCR– based and light microscopy detection. Vet. Parasitol., 20 (3): 249–259.

9. Altay, K.; Aktas, M. and Dumanli, N. (2007): PCR – RFLP analysis of the Tams-1 Gene of Theileria annulata. Turkish Soc. Parasitol. 31 (3): 173–175.

10. Baldwin, C.L.; Malu, M.N.; Kinuthia, S.W.; Conrad, P.A. and Grootenhuis, J.G. (1986): Comparative analysis of infection and transformation of lymphocytes from African buffaloes and Boran cattle with Theileria parva subsp. Parva and Theileria parva subsp. Lawrencei.Infection and immunity j. Vol. (53) No. (1), pp. 186-191.

11. Bishop, R.; Sohanpal, B.; Kariuki, D.P.; Young, A.S.; Nene, V.; Baylis, H.; Allsopp, B. A.; Spooner, P. R.; Dolan, T.T. and Morzaria, S.P. (1992): Detection of carrier state in Theileria parva infected cattle by the polymerase chain reaction. Parasitology, 104: 215-232.

12. De Kok, J. B.; D'Oliveria, C. and Jongejan, F. (1993): Detection of the protozoan parasite Theileria annulata in Hyalomma ticks by the polymerase chain reaction. Exp. Appl. Acarol., 17, 839–846.

13. Dolan, T.T. (1989): Theileriosis a comprehensive review. Rev. Sci. Tech. Off. Int. Epizoot., 8: 11-16.

14. D'Oliveira, C.; van der weide, M.; Habela, M. A., Jacquiet, P. and Jongejan, F. (1995): Detection of Theileria annulata in blood samples of carrier cattle by PCR. J. Clin. Microbiol. 33: 2665–2669.

15. D'Oliveria, C.; van der Weide, M.; Jacquiet, P. and Jongejan, F. (1997): Detection of Theileria annulata by PCR in ticks (Acari: Ixodidae) collected from cattle in Mauritania. Exp. App. Acarol.

16. Gubbels, M.; D'Oliveira, C. and Jongejan, F. (2000): Development of an Indirect Tams-1 enzyme linked immunosorbent assay for diagnosis of Theileria annulata infection in cattle. Clin. Diag. lab. Immunol, pp. 404-411.

17. Gubbels, M. J.; Katzer, F.; Shiels, B. R. and Jongerjan, F. (2001): Study on Theileria annulata population structure during bovine infection and following transmission to ticks. Parasitol., 123: 553–561.

18. Jabbar, A.S.; Shayan, P.; Hugel, F.U.; Biermann, R.; Ewald, CH.; Schein, E. and Gerder, J. (1997): Macroschizonts of Theileria annulata as a vaccine and diagnostic tools. Trop. Anim. Health. Prod. 29: 128–132.

19. Jongejan, F.; de Kok, J. B.; van der Weide, M.; and d' Oliveira, C. (1995): Detection of Theileria annulata infection in carrier cattle and Hyalomma ticks by PCR and development of an ELISA based on a recombinant 30–kDa merozoite surface antigen. In: European Union Third Coordination Meeting on Tropical Theileriosis. Spooner, R. and Campbell, J. (Ed), the Roslin Institute (Edindurgh), Roslin, Midlothian, Scotland, pp. 59–63.

20. Katzer, F.; Mc kellar, S.; Ferguson, M. A. J.; D'Oliveira, C. and Shiels, B. R. (2002): A role for tertiary structure in the generation of antigenic diversity and molecular association of the Tams – 1 polypeptide in Theileria annulata. Mol. Biochem. Parasitol. 122: 55 – 67.

21. Kirvar, E.; Ilhan, T.; Katzer, F.; Hooshmand–Red, P.; Zweygarth, E.; Gerstenberg, C.; Phipps, P. and Brown, C. G. (2000): Detection of Theileria annulata in cattle and vector ticks by PCR using the Tams – 1 gene sequences. Parasitol., 120: 245–254.

22. Murat, G.; Tunay, K.A.; Galp, K.; Emine, A. and Gulcan, E.A. (2008): Serum sialic acids, total antioxidant capacity and adenosine deaminase activity in cattle with theileriosis and anaplasmosis. Bull Vet. Inst. Pulawy, 52: 227-230.

23. Nancy, M. F. B. (1996): Statistical Methodology: I. Incorporating the Prevalence of Disease into the Sample Size Calculation for Sensitivity and Specificity. Acad. Ernerg. Med. 1996; 3:895-900.

24. Shiels, B. R.; Fox, M.; Mckeller, S.; Kinnaird, J. and Swan, D. (2000): An upstream element of Tams-1 gene in a site of DNA–protein interactions during differentiation to the merozoite in Theileria annulata J. Cell Sci.113: 2243–2252.

25. Stagg, D.A.; Young, A.S.; Letich, B.L.; Grootenhuis, J.G. and Dolan, T.T. (1983): Infection of mammalian cells with theileria species. Parasitol, 86: 243-254.

26. Thrusfield, M. (2005): Veterinary epidemiology 3rd Edition, Blackwell science publishing, Chapter 17, pp.313-317.

27. Vantansever, Z. and Nalbantoglu, S. (2002): Detection of cattle infected with *Theileria annulata* in fields by nested PCR, IFAT and Microscopic examination of blood smears. Tur. J. Vet. Anim. Sci., 26: 1465-1469.