Comparative diagnostic studies on *Babesia* and *Theileria* among cattle at Minufiya province

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Abstract

A total of 405 cattle of different ages, sexes, and breeds were examined clinically for diagnosis of babesiosis and theileriosis. Blood film examination revealed that 98 out of 405 animals showed either *Babesia* organism or the intraerthrocytic stage of *Theileria* inside the red blood cells (RBCs). 33 (8.1%) smear and 65 (16.05%) were positive for *Babesia* and *Theileria*, respectively. By examination of 158 serum samples by IFAT for detecting the presence of antibodies against either *Babesia* or *Theileria* species, 25 samples (15.8%) were positive for *Babesia* antibodies and 33 serum samples (20.9%) were positive for *Theileria* antibodies. For confirming all above methods of diagnosis, we had been used PCR in which 275 blood samples were examined after DNA extraction for the presence of either *Babesia* or *Theileria*. The results revealed that positive samples for *Babesia* were 24 sample (8.73%) and 60 sample (21.82%) were positive for *Theileria*. By comparing the different methods of diagnosis, we found that out of 158 cattle examined by three methods 12 samples were positive for blood parasites by three methods of diagnosis (Direct smear, IFAT, PCR), 45 samples were positive by PCR and direct smear, 4 samples positive by PCR and IFAT, 4 sample was positive by PCR only, and 41 samples positive by IFAT only.

Introduction

Cattle are considered as one of the most important animals as they provide human consumption with meat and milk. These products mainly depend on the general health condition which is affected by infection with several diseases such as bacteria, virus, and parasites as blood parasites **(Young *et al.,* 1988)**.

Babesiosis and theileriosis are from the gravest diseases influencing cattle. These diseases frequently plagued the veterinary authorities, essentially they are sometimes appear as vague problems intervened with other disease conditions. Furthermore, blood parasites cause mortalities, abortion, loss of heat, drop in milk production, and emaciation. In particular, these diseases are severe among foreign breeds that are imported **(Yin, et al., 1997)**.

The examination of Giemsa-stained blood smears was the method of choice to detect *Babesia* in blood of infected animals especially in acute cases. It was necessary to develop serological techniques to detect the circulating specific antibodies against blood parasites especially in the very early and chronic stages of the disease and in subclinical infection. The antibodies tend to disappear in this carrier state where as piroplasms persist. Therefore, the application of new diagnostic techniques such as PCR is necessary to detect these carriers **(Rania, 2009)**. In addition serological diagnosis by indirect immunoflourescence antibody test can be used for detection of antibodies against *Theileria* species **(El-Ghaysh, 1993)**.

The fundamental goal of the present work can be summarized on the following:

1. Determination of the incidence of *Babesia* spp. and *Theileria* spp. Infection among cattle by different methods of diagnosis such as Giemsa stained blood film, serological tests (IFAT), and PCR.

2. Figure out the relationship between the results of these different methods of diagnosis.

Materials and Methods

I. Materials:

1. Animal:

A total of 405 cattle of different ages, sexes, and breeds were examined clinically in the field for diagnosis of the animals which suffered from signs of blood parasite such as babesiosis and theileriosis. Blood smears and blood samples were collected to confirm clinical diagnosis and detect carrier animals. Those animals were examined during field trips in Minufiya province.

2. Samples:

2.1. Blood smear:

Clean dry slides were used for collection of blood smears from diseased and apparently healthy cattle. Totally 405 blood smear were collected.

2.2. Blood samples:

275 blood samples were collected in anticoagulant (sodium salt of EDTA) coated tubes of 3 ml volume using sterile syringe with wide bore for DNA extraction to be examined by PCR. These samples were frozen at -20 °C till use. Other 275 blood samples were collected without anticoagulant using sterile needles with wide bores and let to clot for obtaining non hemolyzed clear serum for serological examination by IFAT.

3. Materials used for blood smear examination:

These materials include clean glass slides, methyl alcohol, and Giemsa stain stock solution.

4. Materials used for IFAT:

A. Coated slides for *Babesia* and *Theileria* spp: Slides coated by Babesia antigen and others coated with Theileria antigen were kindly obtained from Veterinary Serum and Vaccine Research Institute (VSVRI) and used as antigen for detecting the positive serum samples by indirect IFAT.

B. Conjugate: Fluorescein labeled (FITC) affinity purified antibody to bovine IgG was developed in rabbit (Sigma immuno chemicals co.). This conjugate reacts specifically with bovine IgG. The conjugate was diluted 1:16 in Phosphate buffered saline (PBS) and gave positive reaction with positive serum samples contain antibodies either for Babesia or Theileria only.

C. Phosphate buffered saline (PBS) pH 7.2: It was prepared according to (Pollard, 1997).

D. Glycerol buffer (pH 8.3): Glycerin ……………………45 ml
                                     $0.1M$ NaHCO3……………...…5 ml

E. Florescent research microscope: It was used to detect positive results in which anti bovine IgG conjugated with florescence appears. It has a fixed camera by which can take on site photos. It is of model jenalumar.

5. Materials for PCR:

A. Materials for DNA extraction (Manual chemical method): They include cell lysis buffer (pH8.0), ethanol, isopropanol, potassium acetate solution, red blood cell lysis buffer and proteinase K (20mg /ml).

B. Materials used for Polymerase Chain Reaction (PCR):

a. DNA 2X Master Mix: Master Mix ready to use was obtained from Fermentas, Germany. It contains Taq DNA polymerase (in reaction buffer 0.05 unites/µl), MgCl2 4 mM, and dNTPs (dNTP, dCTP,dGTP, dTTP) 0.4 mM of each.

b. **Primers:** The primers were synthesized by Bioneer Company, Germany. The sequences of the primers used are common for Babesia and Theleria (GenBank accession no. EF185818).

Table 1: The primers sequences used for PCR for both *Babesia* and *Theleiria*

Primers	Sequences
Forward primer	TTG TAA TTG GAA TGA TGG
Reverse primer	CCA AAG ACT TTG ATT TCT CTC

c. **Programmable thermal controller (PCR machine):** Thermal Cycler-Biometra T personal/ S/N 1205334 was used for amplification of DNA.

6. **Reagents used for electrophoresis**:

A. **Agarose:** Agarose was purchased from International New Technologies in New Hoven, Connecticut, USA. This agarose was DNase and RNase free and prepared as 1.5% concentration.

B. **TBE buffer:** Tris Boric acid EDTA buffer (pH 8) was prepared from Tris Hcl, EDTA and boric acid according to (Sambrook and Russel 2001).

C. **Loading buffer:** Bromophenol (1%) was used for depositing PCR products in the gel wells and was prepared as 6 X concentrations. It was supplied by Fermentas, Germany.

D. **Molecular marker (DNA marker):** DNA molecular weight marker was obtained from Fermentas as 100 bp DNA ladder.

E. **Ethidium bromide:** (2.7 Diamino 10 ethly - 9 phenyl phenathridium bromide, Applichem) 10mg/ml of distilled water was used to stain DNA during examination by UV illumination.

II. **Methods:**
1. **Preparation of blood film:** Thin blood films were smeared according to **(Coles, 1986)**.
2. **Examination of blood film for *Babesia* and *Theileria*:** Smears were examined about ¼ - ½ inches from the end of the film and transversed from one side of the film to other (cross-sectional-method) to give constant and representative samples according to (Barrent, 1977). Each slide was examined twice before being considered negative.
3. **IFAT procedure:** The IFAT was performed on examined serum samples by a modified IFAT based on the technique described by (Leeflang and Perie 1972).
4. **DNA extraction:** Manual chemical method (rapid isolation of mammalian DNA) was applied. This technique was done according to (Sambrook and Russell 2001).
5. **DNA amplification (PCR):** The DNA samples were tested in 50 µl reaction volume. The tested samples were mixed with PCR components according to the following amounts:

Substance	volume	final concentration
DNA	variable	1µ g
2Xmaster mix	25µ l	1x
Forward Primer	variable	0.1-1µM
Reverse Primer	variable	0.1-1µM
Deionized H2O	completed to	50 µ l

6. **PCR Protocol:** Primary denaturation: at 95 °C for 5 min which followed by 40 cycles of each follwing steps. Step 1 (Denaturation): at 95 °C for 1 min. Step 2 (Annealing): at 55 °C for common primer for 1 min. Step 3 (Extension): at 72 °C for1 min. Followed by final extension at 72 °C for10 min. Then the product was kept at 4 °C till examination.
7. **Screening of PCR products:** 1.5 gm of agarose was dissolved in 50 ml of TBE buffer (electrophoresis buffer) and melted by heating. 0.5 µg ethidium bromide/ml of agarose was added. Then the agarose was poured in the gel tray. Then, the tray was inserted in the electrophoresis cell containing TBE buffer. 10 µ l of each PCR product mixed with 2 µl loading buffer were applied. Also, 5 µ l of molecular marker was applied. The electrophoresis cell was adjusted at 10 volt/cm. Then, the gel was examined under short wave UV

transilluminator and the results were photographed.

8. **Statistical analysis:** Obtained data were statically analyzed to illustrate the results of his study using the methods of (Norman and Baily, 1997).

Results

1. Detection of *Babesia* and *Theileria* infection in cattle by examination of Giemsa stained blood smears:

A total 405 cattle blood samples were examined by Giemsa stained blood smears at Minufiya province. The results showed that 98 animals showed either *Babesia* or intraerthrocytic stage of *Theileria* inside RBCs. Moreover, 33 (8.1%) smears were positive for *Babesia* and 65 (16.05%) were positive for *Theileria*.
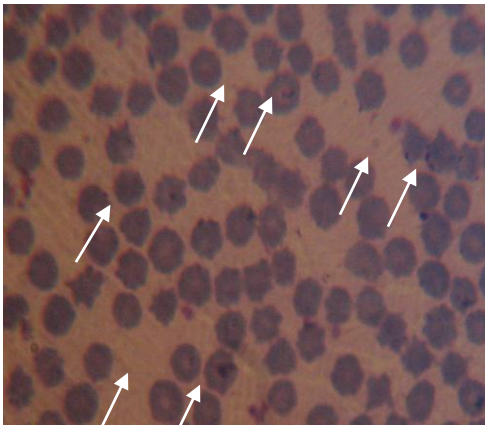
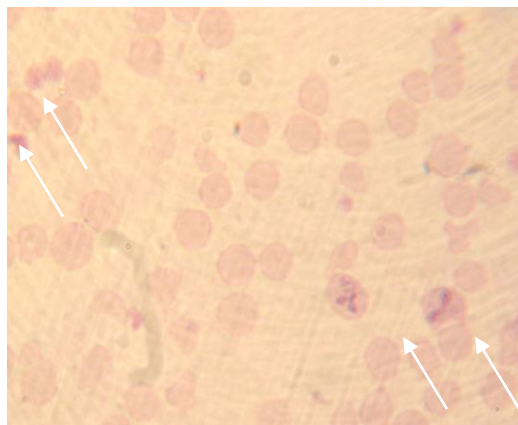**Fig. 1**: Showing *Theileria* protozoan parasite of different shapes inside red blood cells.

Fig. 2: Showing *Babesia* protozoan parasite of different shapes inside red blood cells

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2. Seroprevelance of *Babesia* and *Theileria* species in cattle by examination of serum samples by IFAT:

Total 158 cattle serum samples where examined for presence of antibodies against either for *Babesia* or *Theileria* species. Among them 25 (15.8%) samples were positive for *Babesia* antibodies and 33 (20.9%) samples were positive for *Theileria* antibodies.
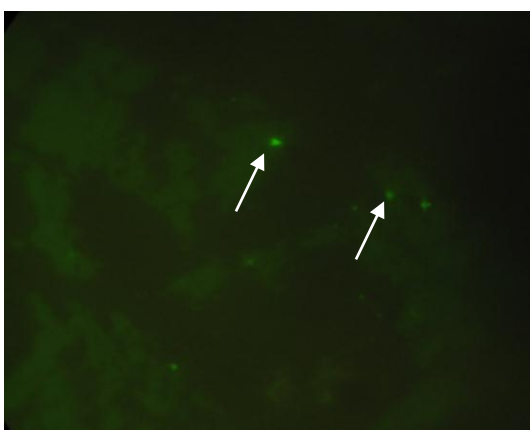

Fig. 3: Showing Typical yellowish fluorescence of *Theileria* piroplasms (arrow) indicating positive reaction in IFAT (100×).
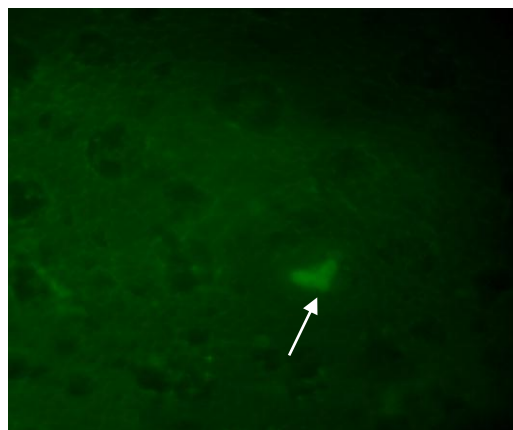

Fig. 4: Typical greenish yellow fluorescence of *Babesia* piroplasms (arrow) indicating positive reaction in IFAT (100×).

Table 2: Seroprevelance of *Babesia* and *Theileria* species in cattle by examination of serum samples by IFAT.

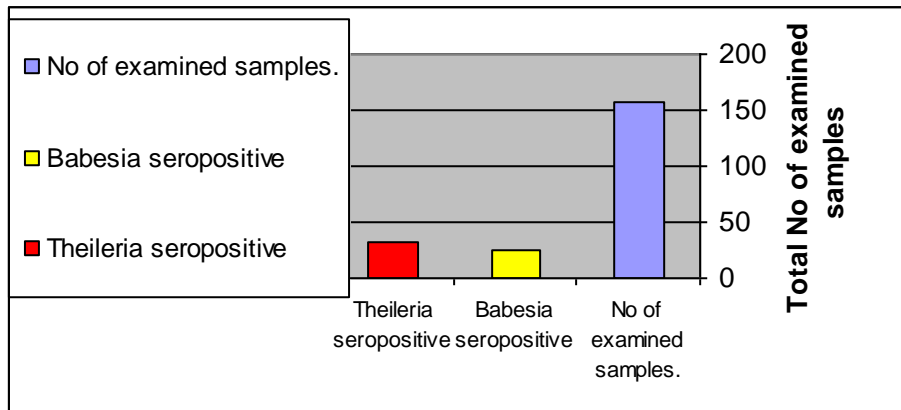| No. of examined samples.	*Babesia* seropositive		*Theileria* seropositive	
	+ve	%	+ve	%
158	25	15.8	33	20.9

Fig. 5: Seroprevelance of *Babesia* and *Theileria* species in cattle by examination of serum samples by IFAT.

3. Examination of blood samples by PCR:

A Total 275 cattle blood samples were examined by PCR for the presence of blood parasite either *Babesia* or *Theileria*. The results revealed that positive samples for *Babesia* were 24 (8.73%) sample and 60 (21.82%) were positive for *Theileria*.
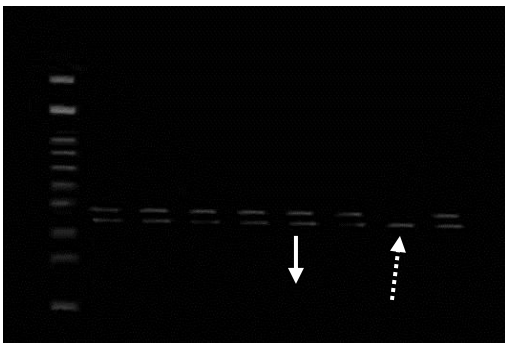

Fig. 6: PCR result showing the presence of bands at 350pb for *Babesia* (discontinuous arrow) and 370pb for *Theileria* (linear arrow) in the pooled samples.

Fig. 7: PCR result showing the presence of bands at 350pb for *Babesia* (discontinuous arrow) and 370pb for *Theileria* (linear arrow) in the pooled samples.
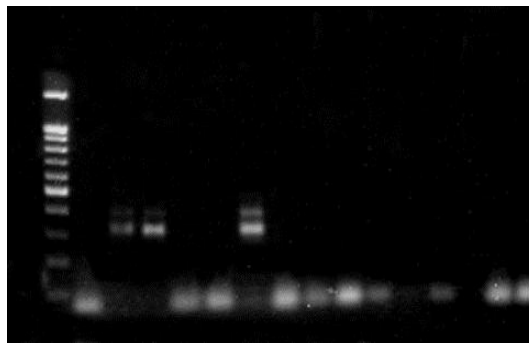
Fig. 8: PCR showing positive separate samples of positive pool at 350pb for *Babesia* and 370pb for *Theileria*.
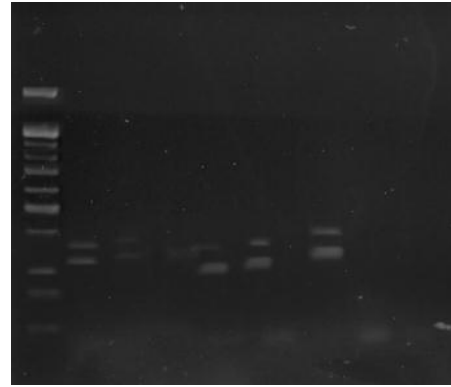

Fig. 9: PCR showing positive separate samples of positive pool at 350pb for *Babesia* and 370pb for *Theileria*.

4. Rate of mixed infection:

The results revealed that mixed infection (both *Babesia* and *Theileria* infection) using Giemsa stained blood smears and PCR were 11(2.7%).

Table 3: Incidence of *Babesia* and *Theileria* in cattle by examination of blood samples by PCR.

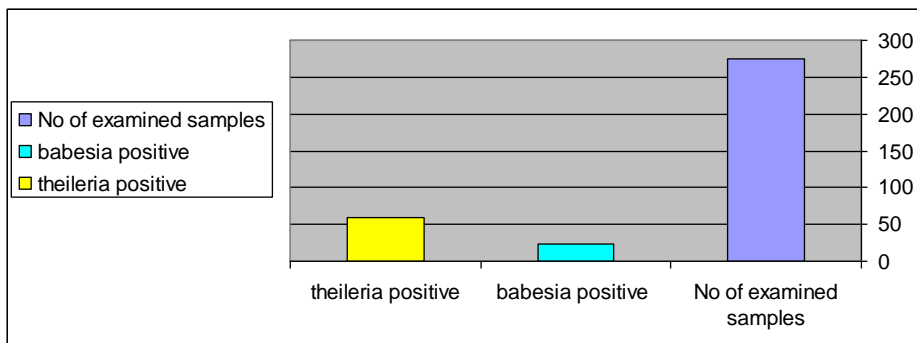| No of examined samples	*Babesia* positive		*Babesia* positive	
	+ve	%	+ve	%
275	24	8.73	60	21.82

Fig. 10: Incidence of *Babesia* and *Theileria* species in cattle by examination of blood samples by PCR.

5. Correlation between different methods used for diagnosis of blood parasite:

By examination of 158 samples by three methods of diagnosis (direct smear, IFAT, and PCR), the results showed that 2 samples were positive for babesiosis by the three methods of diagnosis (direct smear, IFAT, PCR), 16 samples were positive by PCR and direct smear, 2 samples positive by PCR and IFAT, 1 sample was positive by PCR only, and 21 samples positive by IFAT only.

In case of the diagnosis of theileriosis by the three methods, the results showed that 10 samples were positive by three methods of diagnosis, 29 samples were positive by PCR and direct smear, 2 samples were positive by PCR and IFAT, 3 samples were positive by PCR only, and 21 samples were positive by IFAT only.

Total positive samples by different methods of diagnosis were 42 (26.6%) for *Babesia* and 65 (40.41%) for *Theileria*.

Table 4: Incidence of *Babesia* and *Theileria* species in cattle by examination of blood samples by direct microscope, IFAT, and PCR.

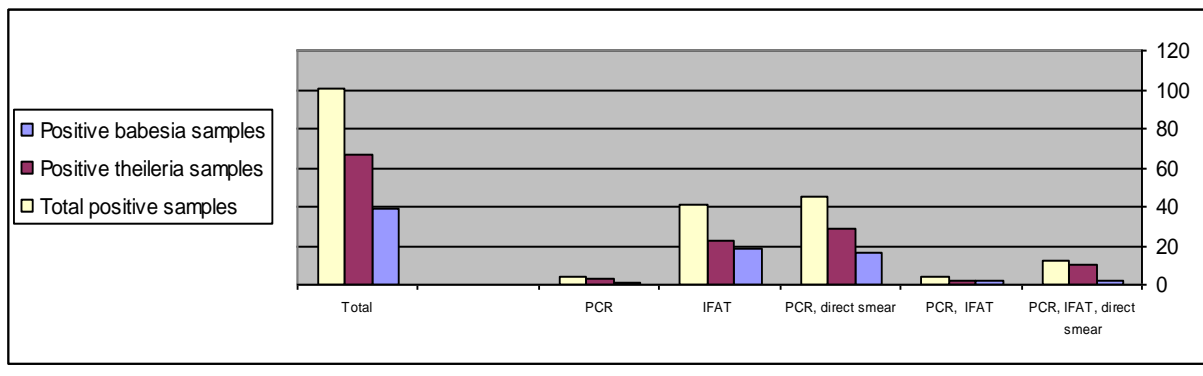| No of examined samples	Positive babesia samples	Positive theileria samples	Total positive samples
PCR, IFAT, direct smear	2	10	12
PCR, IFAT	2	2	4
PCR, direct smear	16	29	45
IFAT	21	21	42
PCR	1	3	4
Total	42	65	107

Fig. 11: Incidence of *Babesia* and *Theileria* species in cattle by examination of blood samples by direct microscope, IFAT, and PCR.

Discussion

Babesiosis and Theileriosis are from the most important protozoal diseases influencing cattle. In particular, these diseases are severe among foreign breeds **(Yin, et al. 1997).**

In the present study, the result obtained from Gimesa stained blood smears revealed that 33 smear (8.1%) were positive for *Babesia.* This nearly similar to the results obtained by **(Chafick, 1987; and Abdel gawad, 1993)** in Egypt, and **(Sevnc et al., 2001)** in turkey. On other hand, these results were disagreed with the results obtained by **(Osaki et al., 2002)** in Brazil who reported that the infection rate was about 64%. Also, the results shown here are in disagreement with those obtained by **(Jeon, 1978)** in Korea who reported that the infection rate was about 23%. This disagreement might due to the variation of environmental conditions (temperature and humidity) which affect both parasites and vectors.

The result of Gimesa stained blood smears for *Theileria* in the present study revealed that 65 smears (16.05%) out of 405 were positive. This result was agreed with the result obtained by **(Jeon, 1978)** in Korea which were 17%, also agreed with result obtained by **(Acici, 1995)** in Samsun region who reported that out of 149 examined samples 26 (17%) were postive for *T. annulata*. **Abu EI-Magd (1980)** in Quena province reported that the infection rate of 11.1% among cattle was *T. annulata* which nearly similar to our result. **Adel (2007)** in Gharbia province reported that the infection rate was 11.31% among cattle were positive *T. annulata*. These results were agreed with our results. However, the results shown here are in disagreement with that obtained by **(Gamal EI-Dien, 1993)** in EI-Behera province where the incidence of *T. annulata*

was 65.4% by stained blood films. Also, our results showed disagreement with that obtained by (**EL bahy, 1986**) in Egypt who reported an infection rate for *T. annulata* of 65% and 53% in cattle and buffaloes. **Schoepf et al., (1984)** in Somalia showed that 49.5% cattle were positive for *Theileria* spp. by blood smear. These results are completely different from our results. These disagreement may be either due to different environmental condition or time of examination.

The present study revealed that mixed infection using Gimsa stained blood smears were 11 (2.7%) showed mixed infection of *Babesia* and *Theileria*. These result were agreed to that obtained by (**Dumanli and Ozer, 1987**) in Turkey who found that the infection rate of mixed infection in cattle for *B. bigemina* and *T. annulata* were (1.5%).

Also by examining 158 cattle serum samples for presence of *Theileria* antibodies using IFAT, the result revealed that 33 (20.9%) samples were positive. This result showed agreement with that obtained by (**Sayin et al., 2003**) who recorded that 34 cattle out of 155 (21%) seropositive for *T. annulata*. Also the resuts is in agreement with the results mentioned by (**Adel, 2007**) in Gharbia province who reported that the peak of the incidence of *T. annulata* seropositive animals using IFAT were 27.8% and 22% among the native breed cattle examined in spring and summer, respectively. Also, the result is agreed with the results reported by (**Salem, 1998**) who recorded that the highest incidence of *T. annulata* by of IFAT was in Bassiun where the incidence was 25.71%. Our result showed disagreement with that obtained by (**Gamal EI-Dien, 1993**) in EI-Behera Province who recorded that the incidence of *T. annulata* was 71.9%. Also results showed difference with that recorded by (**Caille, 1987**) in Somalia who found that about 71.2% of cattle had antibodies against *T. annulata*. This disagreement may be due to difference in animal species susceptibility and also may due variance of animal locality.

By examination of 275 samples for *Babesia* infection using PCR we found that 24 (8.73%) were positive. These results are similar to that mentioned by (**oliveria et al., 2005**) who recorded that 10% of cows were positive for *Babesia* using PCR. Also, the results are similar to the results obtained by (**Figuora et al 1993; and Gubbels et al., 2002**). However, our results did not agree with the results obtained by (**devos, 1994**) in France who found that the prevalence rate of *Babesia* spp in cattle was 20%. Also, our results did not agree with result mentioned by (**Mohamed, 2008**) who detected 8 out of 30 cattle representing 26.7% were positive for *Babesia* infection using PCR. Also, the results shown here were disagreed with results obtained by (**Rania, 2009**) who mentioned that rate of infection for *Babesia* was 25.33%. This disagreement may be due to locality differences or the deifference may be due to the other authers might examine diseased cases only but we examine all animals either clinically affected or not.

By examination of the same samples we found that infection rate of *Theileria* using PCR was 21.8%. These results are similar to those mentioned by (**Aktas et al., 2005, Altay et al., 2007**). Also, our results agreed with result obtained by (**Ogden et al., 2003**) who found that 23.4% of cows positive for *Theileria* by PCR in Tanzania.

By comparing between three methods of diagnosis we found that the three methods were different in result but we should say that IFAT detect the presence of antibody not for antigen. Therefore, it is not sensitive technique in diagnosis of acute cases but can give us an idea about epidemiology of the disease and its prevalence. On the other hand, the best and rapid method is direct microscopic smear which is till now the method of choice for diagnosis of blood parasites in the field but it had a limitation which is not all negative cases by direct smear are really negative. This was confirmed by PCR which gave 8 animals more positive than direct smear. Therefore, we can say most accurate and sensitive method for diagnosis of blood parasite is the PCR but most rapid one was direct smear and the best one give an idea about immunity post infection is IFAT.

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الملخص العربي

تمثل الأبقار مصدر هام لإنتاج اللحوم والألبان في مصر ولأن أمراض طفيليات الدم تسبب خسائركبيرة نتيجة التأثير علي صحة الحيوان من ضعف عام وهزال وقلة انتاج اللبن فقد قمنا بإجراء هذة الدراسة.

أجريت الدراسة للمقارنة بين الطرق التقليدية والطرق الحديثة في تشخيص طفيليات الدم في الأبقار. أظهر فحص شرائح الدم أن نسبة الأصابة كانت ٩٨ حالة (٢٤٪) كان منهم ٣٣حالة اصابة بالبابيزيا (٨٪) و٦٥حالة إصابة بالثايليريا (١٦٪). بفحص مصل الدم من عدد ١٥٨ حيوان بإستخدام الأختبار الفلورسيني المضئ وجد ان الأجسام المناعية المضادة للبابيزيا موجودة في عدد ٢٥ عينة (١٥,٨٪) وكانت الأجسام المناعية المضادة للثايليريا موجودة في ٣٣عينة (٢٠,٩٪). باستخدام اختبار البلمره المتسلسل استخدمنا عينه دم من ٢٧٥ حيوان بعد فصل ال دي إن ايه (المادة الوراثيه) الخاص بطفيل الدم وكانت النتيجة وجود ٢٤ عينه ايجابية للبابيزيا (٨,٧٣٪) وعدد ٦٠ عينه ايجابية للثايليريا (٢١,٨٢٪) .

بمقارنه الطرق التشخيصية المختلفة والتي استخدمت في ١٥٨ عينه فقط كانت النتائج كالتالي: ١٢ عينه كانت موجبه بالثلاث طرق، عدد ٤٥ عينه موجبه باستخدام اختبار البلمره المتسلسل وشرائح الدم، ٤ عينات موجبه باستخدام الاختبار الفلورسيني المضئ واختبار البلمره المتسلسل معا، ٤ عينات موجبه باستخدام اختبار البلمره المتسلسل وحده، وأخيرا ٤١عينه موجبه باستخدام الاختبار الفلورسيني المضئ وحده.