Egyptian Veterinary Medical Society of Parasitology Journal

Original Article

Comparative study for diagnosis of babesiosis and theileriosis in different age groups of cattle in some localities in Egypt with treatment trials

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Abstract:

Babesia bovis, Babesia bigemina, Theileria annulata are the most important endemic blood parasites in Ismailia and Sharkia Provinces, Egypt, which transmitted by ticks causing babesiosis and theileriosis which characterized by fever, enlarged lymph nodes, pale mucous membranes and may lead to death. Therefore, the aim of our study is to determine the infection rates of *B. bovis*, *B. bigemina* and *T. annulata* in relation to age. We found that the rates by staining method were as the following: 12.90%, 6.45%, 38.71% and 4.84% for *B. bovis*, *B. bigemina*, *T. annulata* and mixed infection, respectively, while by polymerase chain reaction (PCR) the rates were 35%, 20%, 60% for *B. bovis*, *B. bigemina* and *T. annulata*, respectively. This study confirmed the incidence of infection with *Babesia and Theileria species* in relation to age group of animals was 77.78% in adult age group, 66.67% in less than 3 months age group and 53.19% in 3-6 months age group. Also, our study proved that the use of imidocarb and buparvoqune beside acaricides considered a good choice for treatment. Although our results proved that using of specific primers in PCR was a rapid and an accurate method for differential diagnosis between *Babesia and Theileria* species in cattle, we found that the use of staining method was more helpful especially in mixed infection cases. In the end, we recommend using both methods together to get better results.

Keywords: Age, *Babesia*, Egypt, Giemsa, Polymerase Chain Reaction, Tams1, *Theileria*

INTRODUCTION

Babesiosis and theileriosis are the most important haemoparasitic diseases in veterinary medicine. The incidences of these diseases constitute a significant economic loss as they cause a marked decline in the rate of meat, milk production and sometimes death in cattle. In addition, the infected cattle suffered from fever, anemia, haemoglobinurea, jaundice, lethargy and enlarged lymph nodes, especially in the acute stage of infection **Inci et al. (2007).** On the contrary, the infected cattle become asymptomatic in the chronic stages.

When hard ticks attacked cattle to take blood meal, *Babesia species* sporozoites released and transformed into trophozoites inside the red cells. While, *Theileria* sporozoites invaded lymphocytes and then schizogony and merogony were performed **Zintl et al. (2003)**.

In Egypt, *Babesia bigemina* and *Babesia bovis* were the most common causes of bovine babesiosis **Nagati (1947).** While, theileriosis was mainly caused by *Theileria annulata* **El-Ashker et al. (2015).** The recovered animals from acute infection might remained as carriers for the infection **Brown (1990).**

The blood staining method might be insufficient in the detection of infection in carriers, so we found an essential need to diagnose them by using conventional PCR method. The diagnosis of blood parasites by microscopical examination of Giemsa stained blood smears required a lot of effort, especially in the herds of large animals, in addition to the lack of accurate results in the case of light and chronic infection **Calder et al. (1996)** and **Mosqueda et al. (2012),** so it was necessary to use PCR and compare the obtained results with those obtained by microscopical examination of the stained blood smears. So, our study aimed to evaluate the efficiency of Giemsa staining and conventional PCR methods as a rapid and accurate diagnostic tool for detection of *Babesia bovis, Babesia bigemina* and *Theileria annulata* infection in cattle in Egypt. As well as, our study determined the best method for treatment of the infected cattle.

MATERIAL AND METHODS

1. Collection of animals:

Total number of cattle examined was 124 (104 from Wadi Almullak farm at Ismailia province and 20 cattle from different localities admitted to the Veterinary Clinic, Faculty of Veterinary Medicine; Zagazig University, Sharkia Province, Egypt. Examined animals were classified according to the age into three groups:

a) Adult group **≥** three years.

b) Young group less than three months.

c) Young group three-six months (Table 1).

Cattle were clinically examined according to **Rosenberger et al. (1979).**

This study was ethically approved by ZU-IACUC Committee, Zagazig University, Egypt (ZU-IACUC/2/F/5/2018).

1.2. Animal management:

The examined cattle at Wadi Almullak were suffering from low hygienic condition and overcrowding. The animals were collected from different markets beside some imported cases and there was a continuous entry of new cattle to the farm.

2. Collection of the blood samples:

We collected the blood samples in tubes containing EDTA from the tail vein of cattle infested with hard

tick and suffered from increasing in their body temperature. Some animals showed enlargement in prescapular and prefemoral lymph nodes, lacrimation with pale conjunctival mucous membrane, loss of appetite and decrease in their body weights. The collected blood samples kept at -20^0 C until DNA extraction.

2.1. Blood film preparation

We immediately spread thin blood film, then fixed with methanol for 20 minutes and stained with freshly prepared Giemsa stain 25% for one hour. The stained films examined under oil immersion lens to identify and differentiate trophozoites of *Babesia bovis, Babesia bigemina* and *Theileria annulata* according to the characters described by **Soulsby (1982).**

3. Molecular characterization (PCR assay)

DNA extraction from blood samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. Briefly, 200 µl of the blood sample was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56^{O}C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit. The used primers were supplied from Metabion (Germany). They were listed in (Table 2).

Primers were utilized in a 25- µl reaction containing 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an applied biosystem 2720 thermal cycler. The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software. PCR was performed in Animal Health Research Institute, Dokki, Egypt.

4. Treatment trials for babesiosis and theileriosis

Individual cases of cattle suffered from theileriosis were treated with buparvoquone (Avico Company, Jordan) at dose of 2.5 mg/kg intramuscular for two times with 72 hours interval. While, cases suffered from babesiosis were treated with imidocarb dipropionate (Adwia Company, Egypt, 1.2 mg/kg, s/c) plus application of acaricides on cattle skin and surrounding environment. While, in a Wadi Almullak farm; dipping path was absent and the acaricides were randomly sprayed on the skin of infested cattle only and did not apply to the surrounding environment.

RESULTS

1. Clinical findings

The clinical examination of cattle revealed fever (39-41°c), anorexia, pale, icteric and congested mucus membrane (Figure 1a, 1b, 1c and 1d) and nervous signs, those lead to suspect that the infection caused by *Babesia species*. Also, we found enlargement in the superficial lymph node (Fig. 1e), corneal opacity, respiratory manifestation (cough, dyspenea and stretched neck with nasal discharge) and dark red feces, those lead to suspect that the infection was with *Theileria annulata*. In addition, we observed heavy tick infestation in the skin of cattle (Fig. 1f).

2. Infection rate of *Babesia bovis, Babesia bigemina* and *Theileria annulata* in cattle

Microscopical examination of Giemsa stained thin blood smears from 124 cattle revealed that the infection rate was 58.06%. The infection rates were 12.90%, 6.45%, 38.71% and 4.84% for *B. bovis, B. bigemina, T. annulata* and mixed infection (both of *B. bovis and B. bigemina,* and *T. annulata,* Fig. 2j), respectively (Table 3). Regarding the occurrence of *Babesia* species and *Theileria* species in the examined animals, it was 77.78%, 66.67% and 53.19% in adult group, less than 3 months group and 3-6 months group, respectively (Table 4).

3. Morphological identification

During microscopical examination of stained blood films, we recorded three types of blood parasite trophozoites included *Babesia bovis, Babesia bigemina* and *Theileria annulata*. *B. bigemina* trophozoites were large form measuring 4-5.3 µm × 2.0 µm and appeared pear shaped. Each trophozoite had one dark stained spot (nucleus) as shown in (Fig. 2a and 2b). While, *B. bovis* trophozoites were smaller forms measuring 3.0 µm × 1.5 µm and centrally located inside the red cells as shown in (Fig. 2c, 2d and 2e). The two trophozoites made an acute and obtuse angle with each other in B. bigemina and B. bovis, respectively. In addition the red cells infected with *Theileria annulata* showed varied trophozoite forms (comma shaped, ring form or rod shaped) and measuring 0.5 - 1.5 µm as shown in (Fig. 2f, 2g, 2h and 2i).

4. Molecular characterization (PCR assay)

For PCR, the target sequence chosen for amplification was part of the mitochondrial 18s rRNA gene. Those variable regions had shown to be suitable genetic markers for distinguishing *Babesia bovis* and *Babesia bigemina.* The length of their PCR products was 356 bp and 278 bp, respectively (Fig. 3a&3b). While, *T. annulata* amplified segment was 785 bp for Tams1 gene (Fig. 3c). We recorded the infection rates by using PCR and they were 35%, 20% and 60% for *B. bovis, B. bigemina* and *T. annulata*, respectively.

5. Treatment trials for babesiosis and theileriosis

After treatment with buparvoquone for theileriosis and imidocarb for babesiosis, the individual cases showed rapid response after treatment, while cattle treated at a Wadi Almullak farm did not show clear improvement at the status and new cases of blood parasite infections were recorded.

Table 1. Different cattle age groups examined at Ismailia and Sharkia provinces

Location	Number of cattle examined	Age group		
		1-3 month	3-6 month	≥ 3years
Ismailia Sharkia Total	104 20 124	10 2 12	94 - 94	- 18 18

Table 2. Primers sequences, target genes, amplicon sizes and cycling conditions

Parasite name	Primers sequences (5' →3')	Amp. seg. (bp)	1st De.	Amplification			F. Ex.	Ref.
				2nd De.	An.	Ex.		
Babesia bovis	BoF 5'CACGAGGAAGGAACTACCGATGTTGA3'	356	94˚C 5 min.	94˚C 1min	55˚C 1min	72˚C 1min	72˚C 10 min.	**Figueroa et al. (1993)**
	BoR 5'CCAAGGAGCTTCAACGTACGAGGTCA3'							
				35 cycles				
Babesia bigemina	BilA 5'CATCTAATTTCTCTCCATACCCCTCC3'	278	94˚C 5 min.	94˚C 1min	55˚C 1min	72˚C 1min	72˚C 10 min.	**Figueroa et al. (1993)**
	BilB 5'CCTCGGCTTCAACTCTGATGCCAAAG3'							
				35 cycles				
Theileria annulata	Tams1F 5'ATGCTGCAAATGAGGAT3'	785	94˚C 3 min.	94˚C 1min	60˚C 1 min.	72˚C 1min.	72˚C 10 min.	**Kirvar et al. (2000)**
	Tspms1R **5'**GGACTGATGAGAAGACGATGAG**3'**			40 cycles				

Amp.: Amplified, seg.: segment, De: Denaturation , An.: Annealing,, Ex.: Extension, F.: Final and Ref.: References

Table 3. Infection rates of Piroplasmosis in cattle infection by:

a- Microscopical examination in cattle

Examined animals number	Total Infected number (%)	Single infected number (%)			Mixed infected number (%)
		Babesia bovis	*Babesia bigemina*	*Theileria annulata*	
124	72 (58.06%)	16 (12.90%)	8 (6.45%)	48 (38.71%)	6 (4.84%)
b- PCR		35%	20%	60%	

Table 4. Infection rates among different age groups of cattle

Age group	Number of animals		% of infection
	Examined	Infected	
Adult ≥3 years	18	14	77.78%
Less than3 months	12	8	66.67%
3- 6 months	94	50	53.19%
Total	124	72	58.06%

Fig. 1: 1a&1b) Cattle showed pale conjunctival mucous membrane, 1c) Congested conjunctival mucous membrane, 1d) Icteric vaginal mucous membrane, 1e) Enlarged prefemoral lymph node, 1f) Heavy infested cattle skin with tick (Digital camera).

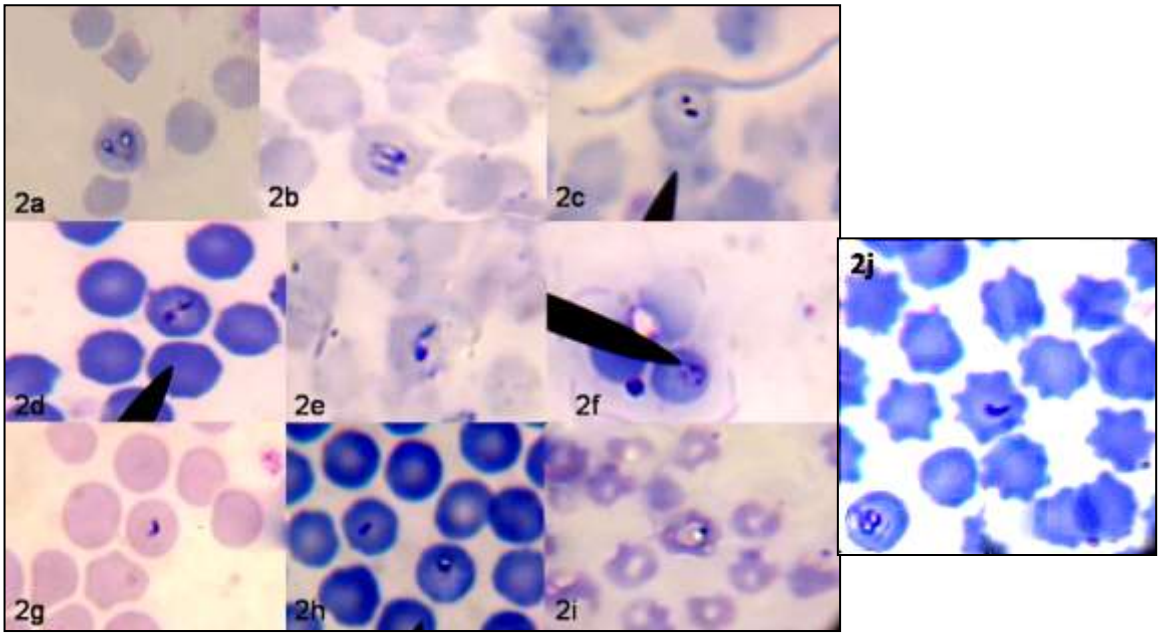

Fig. 2: 2a &2b) Stained blood films showing *Babesia bigemina* trophozoites, 2c, 2d &2e) Stained blood films showing *Babesia bovis* trophozoites, 2f) Comma shaped *Theileria annulata* trophozoite, 2g) Rod shaped *Theileria annulata* trophozoite, 2h &2i) Ring form *Theileria annulata* trophozoite and 2j) Mixed infection (Digital camera).

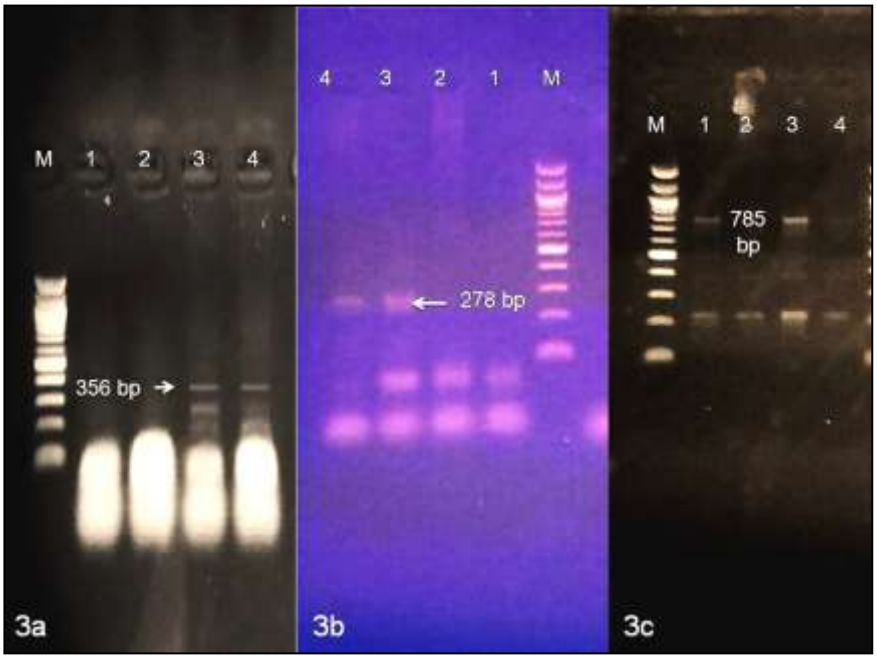

Fig. 3: 3a) Amplified segment (356 bp) for *Babesia bovis*; 1&2: -ve, 3&4: +ve, 3b) Amplified segment (278 bp) for *Babesia bigemina*; 1&2: -ve, 3&4: +ve, 3c) Amplified segment (785bp) for *Theileria annulata*; 1&3: +ve, 2&4: -ve, M: DNA marker.

Discussion

The observed clinical signs in our study were similar to that described by **Rizk et al. (2017).** We suspected that these signs were attributed to the replication of *Babesia species* inside erythrocytes which produced heamolysin and destroy the red cells. This would result in haemoglobnemia, haemoglobnuria, anemia, pale mucous membrane and fever. While, in *Theileria annulata* the replication occurred inside the lymphocytes causing enlarged lymph nodes, lymphocytopenia and jaundice and might death in case of severe pulmonary oedema. Our explanation was also recorded by **El Moghazy et al. (2014)** and **Radostitis et al. (2007).**

Recently, the 18S rRNA genes have been extensively used as suitable targets for the accurate identification of *Babesia species*. The conserved regions of 18S rRNA gene help in designing primers that can be used to amplify the same gene in the related species. Many authors used PCR for identification and differentiation of *Babesia species* affecting cattle. In our study, PCR revealed that the length of PCR products was 356 bp and 278 bp for *B. bovis and B.bigemina*, respectively. This result resembled to that obtained by **Figueroa et al. (1993).** While, the length of the amplified segment was 785 bp for Tams1 gene of *T. annulata*, which was similar to that obtained by **Elsify et al. (2015), Kirvar et al. (2000) and Sivakumar et al. (2012) .**

By Giemsa staining method, we recorded that the infection rate with *B. bovis* was higher than that obtained for *B. bigemina*. They were 12.90% and 6.45%, respectively. In Egypt, **Ibrahim et al. (2013)** mentioned that the infection rates were 5.30% for *B. bovis* and 3.97% for *B. bigemina*, while, **El-Ashker et al. (2015)** recorded their rates as the following 7.3% for *B. bovis* and 1.2 % for *B. bigemina*. On the contrary, **Adham et al. (2009)** reported that the infection rate was higher in *B. bigemina* (14%) than *B. bovis* (6%), while **Al-Hosary (2017)** reported only *B. bovis* infection rate to be 7.9% in Egypt.

By conventional PCR method, we recorded that the infection rates with *B. bovis* was higher than that obtained for *B. bigemina*. They were 35% and 20%, respectively. On the contrary, **Adham et al. (2009)** reported that the infection rate was higher in *B. bigemina* (60%) than *B. bovis* (55%) in Egypt. Lower rates were recorded by **Ibrahim et al. (2009)** to be 17% for *B. bovis* and 9% for *B. bigemina* in Egypt. Also, **Liu et al. (2012)** recorded the lower infection rates in China to be 9.6% for *B. bovis* and 5.4% for *B. bigemina*. **Al-Hosary (2017)** reported the infection rate with *B. bovis* to be 17.1% in Egypt.

By Giemsa staining method, we recorded that the infection rate with *Theileria annulata* was 38.71%.

Nearly similar rates were reported in Egypt to be 30% by **Mahmmod et al. (2010),** 25.37% in India by **Khatoon et al. (2015)** , but lower rates (14.61%) were reported in Egypt by **El Moghazy et al. (2014),** 6.25 % in Iran by **Hoghooghi-Rad et al. (2011) ,** 13% in Egypt by **Ibrahim et al. (2009)**, 12.93% in India by **Kundave et al. (2015),** 16% in India by **Roy et al. (2000),** 10.66% in Iran by **Nourollahi-Fard et al. (2015),** and 8.82% in India by **Sahoo et al. (2017).** On the contrary to our results, the higher rates of infection were recorded in Egypt to be 65.4% by **Gamal El-Dien (1993).**

By conventional PCR method, we recorded that the infection rate with *Theileria annulata* was 60%**.** However, **Mahmmod et al. (2010)** in Egypt and **Khatoon et al. (2015)** and **Kundave et al. (2015)** in India reported that the infection rates were 70%**,** 74.63% and 63.79%, respectively. While, lower rates of infection were recorded as the following: 9.56% in Egypt by **Elsify et al. (2015),** 44% in India by **Roy et al. (2000),** 45.33% in Iran by **Nourollahi-Fard et al. (2015),** 32.35% in India by **Sahoo et al. (2017)** and 9.8% in Sri Lanka by **Sivakumar et al. (2012).**

The difference in infection rates between *B. bovis, B. bigemina* and *T. annulata* was due to the fact that the frequency for detection of infection with *Babesia species* decreased in case of low parasitemias less than 10^{-4} % **Calder et al. (1996).** We returned the differences in infection rates to the geographical, climatic distribution and the system of breeding, in addition to the presence of varied species of hard ticks which played important role in biological transmission of babesiosis and theileriosis.

When we compared our obtained infection rates in different age groups of cattle (77.78% for adult age group, 66.67% for less than 3 months age group and 53.19% for 3-6 months age group) with those obtained by **Kundave et al. (2015) and Utech and Wharton (1982),** we found similarity between the infection rates of adult and 3-6 months age groups. Also, we found that young ages were more resistant to the infection than adult ages. On the contrary to **Kundave et al. (2015),** our results revealed that the infection rate in age group < 3 months was higher and that may be due to entry of newly imported cattle to the farm which might carry different strains of *Babesia* and *Theileria species*. In addition, the imported and crossbred cattle were more susceptible to the infection than the local breeds. This theory had also been proposed by **El Hussein et al. (1991).** Also, we suggest that the improper use of acaricidal agents drive cattle to develop less sufficient immunity against infection and so the maternal immunity will be insufficient to protect their calves from babesiosis and theileriosis.

Our study showed that the treatment trials for individual cases were effective. This result was

agreed with that obtained by **Ganga et al. (2010) and Gharbi et al. (2017).** While, in case of cattle treated at a Wadi Almullak farm showed bad response and so we suggested that this might due to improper application of the acaricides on the cattle skin and low hygienic surrounding environment. This also was similar to that reported by **Rizk et al. (2017).** In addition, the uncontrolled continuous entry of new individuals increases the incidence of reinfection. So we advised the owners in the farm for the regular and proper usage of acaricides inside hygienically prepared dipping path for animals and also for the surrounding the environment.

In conclusion, our study proved that using of specific primers in PCR was a rapid and an accurate method for differential diagnosis between *B. bovis, B. bigemina* and *T. annulata* in blood samples of cattle. Also, our results revealed that the usage of conventional PCR is more efficient than Giemsa staining method for diagnosis of babesiosis and theileriosis. However, the use of staining technique is most helpful in cases of mixed infections with blood parasites. So we recommend using them together to get better results.

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الملخص العربي

دراسه مقارنه لتشخيص مرض البابيزيوسيس و الثيليريوسيس فى الاعمار المختلفة من الأبقار فى بعض المناطق- مصر

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يعد طفيل بابيزيا بوفيس و بابيزيا بايجيمينا وثيليريا انيولاتا من أهم طفيليات الدم المستوطنة في مصر ، والتي تنتقل عن طريق القراد مسببه مرض البابيزيوسيس و الثيليريوسيس و التي تتميز بالحمى وتضخم الغدد الليمفاوية وشحوب الأغشية المخاطية وربما تؤدي إلى الوفاة لذلك فقد أجريت هذه الدراسة لتحديد معدلات الإصابة فى الأعمارالمختلفة من الأبقار وقد كانت على النحو التالي:

بالفحص المجهرى لعينات الدم التى قد تم صبغها باستخدام صبغه جيمسا قد بلغت معدلات الإصابة 12.90 ٪ ، 6.45 ٪ ، 38.71 ٪ و 4.84 ٪ لكل من بابيزيا بوفيس و بابيزيا بايجيمينا وثيليريا انيولاتا والعدوى المختلطة لكل منهم على التوالي بينما بلغت معدلا ت الاصابه باستخدام تفاعل البلمرة المتسلسل (PCR) 35 ٪ و 20 ٪ و 60 ٪ لكل من بابيزيا بوفيس و بابيزيا بايجيمينا وثيليريا انيولاتا على التوالي.

بالإضافة إلى ذلك فقد أوضحت الدراسة حدوث العدوى في الفئات العمرية المختلفة من الأبقار وقد كانت معدلات الإصابة في المجموعه العمرية البالغه 77.78% بينما كانت 66.67٪ في الفئة العمرية الأقل من ثلاثه أشهر وأقل معدلات اصابه تم تسجيلها كانت فى الفئه العمرية من ثلاثة إلى ستة أشهر وبلغت 53.19% .

وقد اكدت الدراسه أيضا ان استخدام عقار الايميدوكارب و البيوبارقوكون بجانب مضادات العنكبيات تعتبر من افضل الطرق المستخدمة فى العلاج.

و بالرغم من أن نتائج الدراسه قد أثبتت أن استخدام تفاعل البلمره المتسلسل باستخدام البريمرات المتخصصة هى طريقة سريعة ودقيقة للتشخيص التفاضلي بين نوعي البابيزيا والثيليريا في الأبقار إلا أن هذه الدراسه قد أثبتت أن استخدام صبغه جيمسا لفحص عينات الدم كان أكثر فائدة خاصة في حالات العدوى المختلطة لذا توصي الدراسه باستخدام كلتا الطريقتين معًا للحصول على أفضل النتائج لتشخيص طفيليات الدم فى الأعمار المختلفة من الأبقار