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Parasitological and molecular identification of *Theileria* Species by PCR-RFLP Method in Sheep, Egypt.

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Abstract

The present study was carried out to investigate the accurate status of ovine *Theileria* infection in sheep at Giza governorate, Egypt during the period from May 2013 to April 2014. A total of 347 sheep blood samples (240 from different flocks and 107 from slaughter houses all over the governorate). Sheep were clinically examined and blood samples were collected on EDETA tubes. Giemsa stained blood smears examination by light microscope showed that 15.56% (54/347) were infected with *Theileria* spp. PCR applied for amplification of a fragment of the 18S ribosomal DNA on 5 positive and 10 microscopically negative samples. 40% (6/15) of examined samples were infected. RFLP assay applied for differentiation of various *Theileria* spp. by using HpaII restriction fermentase enzyme. RFLP results differentiated *Theileria* spp. to *T. lestoquardi, T. ovis* and *T. annulata.* This study concluded that PCR - RFLP is a diagnostic tool enabling direct, highly specific and sensitive identification of *Theileria* spp. when compared with microscopic examination

Keywords: *Theileria, sheep, PCR – RFLP, Egypt.*

Introduction

Ovine theileriosisis a major problem in small ruminants. It is a tick-borne disease caused by apicomplexan parasites of the genus *Theileria*. At least six *Theileria* species have been found to infect small ruminants. Of these, *T. lestoquardi, T. luwenshuni* and *T. uilenbergi* are considered to be highly pathogenic in sheep and goats. The other three species, *T. separata, T. ovis* and *T. recondita*, are generally considered to be non-pathogenic or mildly pathogenic **(Schnittger et al**. **2000; Perston 2001)**. The precise identification of these organisms is essential to understand their epidemiology. Diagnoses of theileriosis in acute cases are based on clinical signs and microscopic examination of blood or organ smears stained with Giemsa or Wright **(Yin et al. 2003)**. These methods are useful in acute cases, but these methods are insufficient for carrier animals. Also, microscopic examination can't do accurate identification of *Theileria* spp. during mixed infections and low parasitemia (**Quintao-Silva and Ribeiro, 2003).** For these reasons PCR has become the preferred method for diagnosis of bovine and ovine theileriosis in epidemiological studies, because this technique is more sensitive and specific than other conventional methods **(Aktas et al. 2005)**. For differentiation of various *Theileria*sp., a RFLP assay was used as a diagnostic tool enabling direct, concurrent, highly specific and sensitive identification of them.**(Jalali et al. 2014).**

Materials and Methods

A total of 347 sheep blood samples were randomly selected from both sexes and different ages (< 1 year,

1-2 year and >2 years) from May 2013 to April 2014 to detect *Theileria* spp. Infection among sheep. Inspected sheep were 240 resident animals from sheep flocks in Giza governorate, Egypt farms and its surrounding villages and 107 from (ElBasatin, El Monibe and El Warrak) abattoirs.

Data was analyzed using SPSS version 20. Pearson chi-square ($\chi 2$) was used to test the relationship between the age, sex, season and animal location with infection percent. Values of $P<0.05$ were considered statistically significant **(Remington and Schork 1970)**.

Samples collection

Blood was collected from jugular vein of 347 sheep (132 male and 215 female). The collected blood samples were obtained from each animal on tubes with anticoagulant (EDTA). The blood samples were used to prepare thin blood smears were prepared for microscopic examination **(Zafar et al. 2006)** and the remaining was stored at -20° C until performing DNA extraction for PCR.

Microscopic examination of blood smears (ME).

The blood smears were air-dried, fixed in methanol, stained with 10% Giemsa stain solution in phosphate-buffer saline (pH 7.2) **(Saal 1964)**. The stained slides were examined with an oil immersion lens at a magnification of 1000x.At least 50 fields were searched per slide before considered negative. For estimating parasitemia, 100 different microscopic fields were carefully examined and the percentage of infected erythrocytes was determined according to **Oura et al. (2004)** as (No of infected RBCS or lymphocyte ÷ total No of RBCS counted) × 100. The size of *Theileria* spp. (piroblasm and schizont) was measured by method described by **Otify (2012**).

Polymerase chain reaction (PCR)

Five positive samples and 10 negative by ME were subjected to DNA extraction using a genomic DNA extraction kit for blood and tissue (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Two pairs of universal screening primers were used to amplify the DNA of all *Theileria* sp. according to **HeidarpourBami et al. (2009).** Primers were forward strand Thei F1 5'-AAC CTG GTT GAT CCT GCC AG-3'and reverse strand Thei R1 5'-AAA CCT TGT TAC GAC TTC TC-3'. The PCR was performed in a total reaction volume of 50 µl containing 3 µl from extracted DNA in concentration 100 ng and 2 µl l of 10xPCR buffer [100 mMTris–HCl (pH 9), 500 mMKCl, 1% Triton X-100], 250 mM of each of the four deoxy nucleotide triphosphates, 1.25 unit Taq DNA polymerase (Promega), and 1 µl from 10 pmol concentration of each primer. The sample tubes were mixed, gently spun and placed in thermocycler .Cycling conditions for PCR were initial denaturation at 95 ^{o}C for 5 min for1 cycle followed by denaturation, annealing and extension for 35 cycles at temperature (95, 54 and72 ^{o}C respectively). At last final extension at 72 ^{o}C for 10 min in 1cycle. PCR products were visualized by UV trans illumination in a 1.5% agarose gel following electrophoresis and stained with ethidium bromide.

Restriction fragment length polymorphism of PCR products (RFLP)

RFLP of PCR products performed in Cairo University, Faculty of veterinary medicine, bio technology department according to **HeidarpourBami et al. (2009)**. RFLP applied for differentiation of various *Theileria* species (*T.annulata, T. lestoquardi* and *T.ovis*). The amplified products were digested with restriction enzymes Hpa II (Fermentas) as described by the supplier. The digestion reaction was set up in 20 µl volume. The digestion mixture consisted of 2 µl of the 10x buffer, 10 µl PCR product and 1 µl (10 U) of Hpa II restriction enzyme made up to 20 µl with autoclaved triple-distilled water. The digestion mix was incubated at 37 ^{o}C for 2 h. The restriction enzyme analysis of the PCR amplified DNA fragments were performed by electrophoresis on the ethidium bromide-stained 2% agarose gel.

Results

Microscopic examination of sheep blood smears showed that 15.56% (54/347) sheep were infected by *Theileria* spp. 17.08% (41/240) and 12.14% (13/107) in resident and pre slaughtered animals from abattoirs respectively (table 1). Parasiteamia among naturally infected sheep was ranged from (0.001- 1%). Monthly and seasonal incidence recorded in table (2). In by monthly incidence the maximum rate of infection was determined in June (28.6%). and the minimum was in November (0%). The highest seasonal incidence of *Theileria* infection was recorded in summer 22.78% and the lowest season was in spring 11.65% and chart (1).

Table (1): Incidence rate of *Theileria* infection between resident and pre slaughtered sheep.

Animals	Examined No	Infected No	% of Infection
Resident	240	41	17.08a
Slaughtered	107	13	12.14a
Total	347	54	15.56

a= refers to the non-significant differences at (p< 0.05).

Table (2): Monthly and seasonal incidence of *Theileria* sp. infection among sheep in Giza governorate.

Month	Examined No.	Infected No.	% of Infection	Season	Examined No.	Infected No.	% of Infection
December	17	3	17.60	Winter	82	14	17. 07
January	17	2	11.70				
February	48	9	18.75				
March	51	6	11.76	Spring	103	12	11.65
April	14	3	21.40				
May	38	3	7.90				
June	35	10	28.60	Summer	79	18	22.78
July	29	5	17.24				
August	15	3	20.00				
September	48	7	14.58	Autumn	83	10	12. 04
October	20	3	15.00				
November	15	0	0				
Total	347	54	15.56%	Total	347	54	15.56

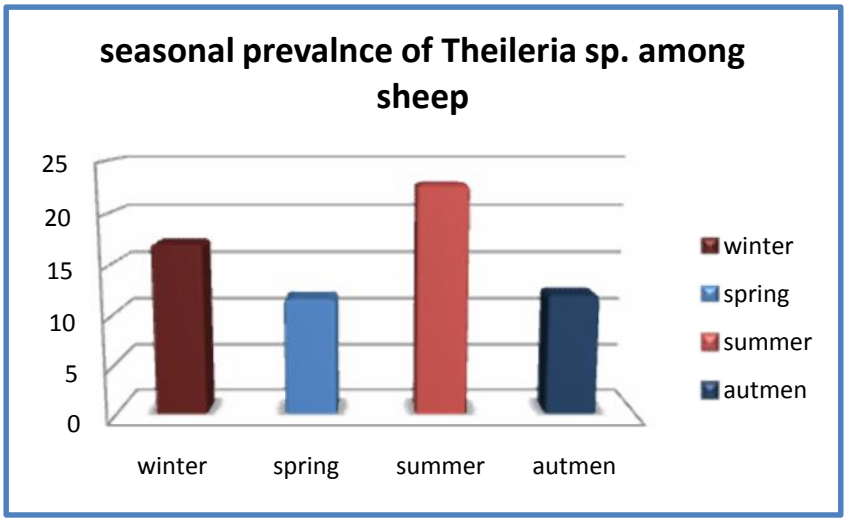

Chart 1: Seasonal Prevalence of *Theileria* Infection among sheep by microscopic examination.

Chi square showed that no significance differences between age and sex effect (table 3) and the percent of infection as the P value was > 0.05.

Table (3): Incidence of *Theileria* infection among different sexes.

Sex	Examined No	Infected No	% of Infection
Male	132	21	15.9a
Female	215	33	15.34a
Total	347	54	15.56

a= refers to the non-significant differences at (p< 0.05).

Different forms of *Theileria* were found in the blood smears (Fig.1) as macro and micro schizonts (51.85% and 14.8% respectively). Its size varied from 8-11µm x 2-5 µm and the chromatine dots varied in size from 1-2.1 µm. Erythrocytic forms were 33.35% which varied in shape from ring to round and *Anaplasma* like with size varied from 1-1.5 µm.

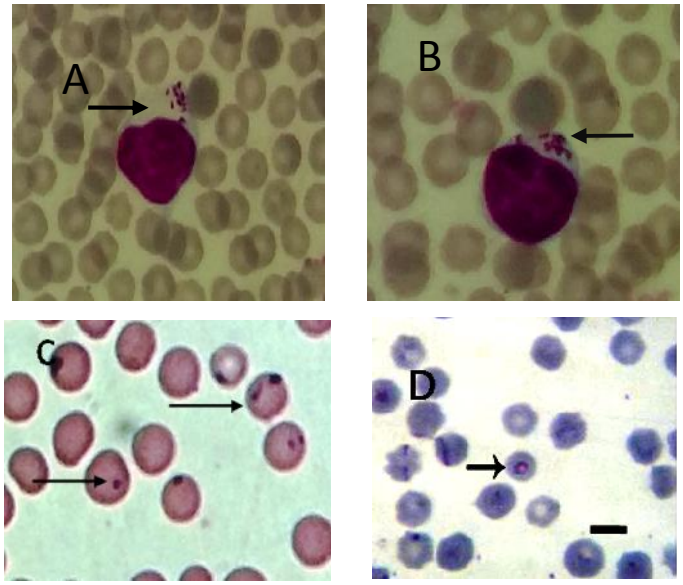

Fig.1: Giemsa stained blood smears, *Theileria* spp. (A) Micro schizont, (B) Macro schizont, (C) Erythrocytic form (comma and anaplasma like shape) and (D) round form.

PCR -RFLP

Investigation of 15 samples (5 positive and 10 were negative in microscopic examination) by PCR using universal *Theileria* species-specific primer sets. The results indicated that all of the samples that were positive on blood smears were also positive via PCR and one from the negative samples by microscopic examination gives positive by PCR (40%). The PCR amplified a mono morphic DNA fragment of 1400 bp size as shown in Fig. (2). restriction fragment length polymorphism (RFLP) of PCR products of the 18S rRNA gene of *Theileria sp.* by Hpa II restriction enzyme was found to differentiate the three *Theileria* spp. presently known in Egypt (*T. ovis, T. lestoquardi and T. annulata*) as shown in Fig.(3) and the digestion pattern recorded in Table (4).

Table (4): The pattern of RFLP of PCR products of different Theileria species by using of HpaII

Theileria sp.	HpaII
T. lestoquardi	900-, 278 and 106bp
T. annulata	1178 and 106bp
T. ovis	856-, 326 and 204bp

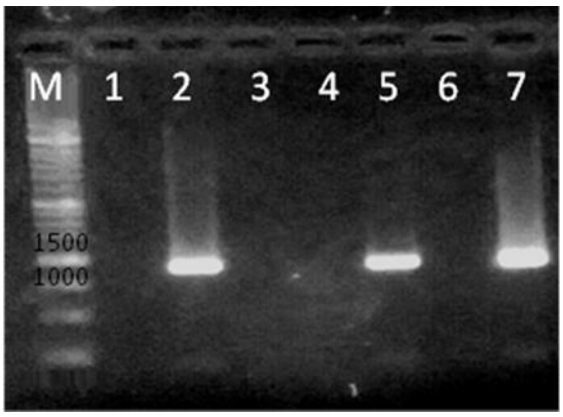

Fig.(2) Agarose-gel electrophoresis of amplication products obtained from *Theileria sp*. M, DNA size marker in bp is indicated at left; lane 2, 5 and 7 *Theileria sp*. positive samples lane 1, 3,4 and6 negative samples

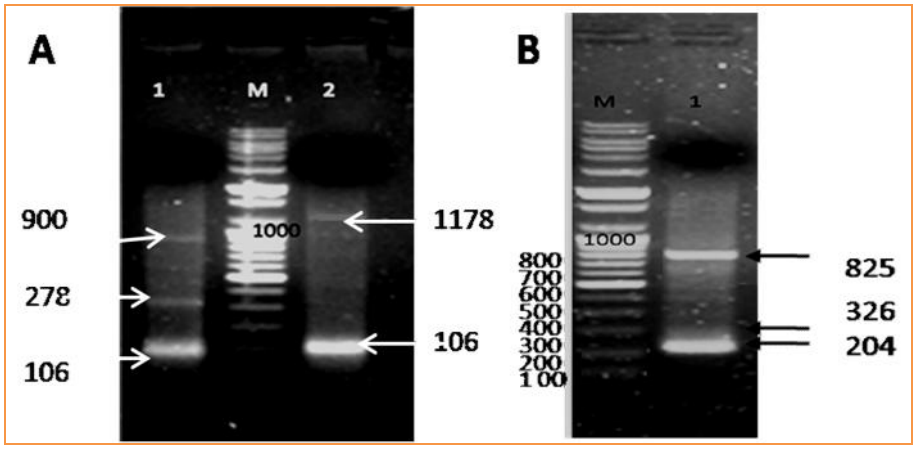

Fig. (3) Restriction digests of *Theileria* sp. amplification products. M, 100 bp DNA size marker (A) Lane 1, *T. lestoquardi* HpaII digest; lane 2, *T. annulata* HpaII digest; (B) M, 100 bp DNA size marker. lane 1, *T. ovis* HpaII digest

Discussion

The present study showed that *Theileria* spp. incidence by microscopic examination was 15.56% (17.08% and 12.14%) in resident and pre slaughtered animals from abattoirs respectively. It is lower than other studies in different location in Egypt as **Hala and El-Kelsh (2006)** detected the prevalence 87.5% and 18.75 % in sheep farms from El-Gharbia and slaughter houses respectively. **Arafaa and Radwan (2007)** found incidence rate in Giza governorate from goats showing clinical signs of theileriosis was 86.1% and 43.1% from apparently healthy one. **Radwan and El Kelsh (2009)** detected the incidence rate in sheep 33.75% and goat 28% in 4 different governorates in Egypt (Elfayoum, Giza, Cairo and El Gharbia)**. Faddel et al. (2010)** detected much higher prevalence (77.93 % of examined sheep and 85.33 % of examined goats) in Halayeb and Shalatin districts. These variations might be regarded to the locations differences and sampling design as the present work was not focused on flocks with known theileriosis history but random selection were occurred.

In Iraq; **Al-Alousi et al. 1988; Al-Amery and Hasso2002**; **Alfetly 2012** and **Dhaim and A'aiz 2013** recorded prevalence of theileriosis in sheep by blood smears to be 19.5%, 33.8%, 26.51% and 22.8 % respectively. In Sudan **Ahmed et al. (2003)** recorded the prevalence rate of *Theileria* infection in resident animals 22.1% and from pre slaughtered animals 17.8%. Also higher prevalence rats were detected in Iran by **Razmi et al. 2003; Razmi and Yaghfoori 2013; Bahrami et al. 2013** and **Jalali et al. 2014** to be 36.17% , 18.6%, 25.35% and 69.7% by Microscopic examination respectively. **Aktas et al. (2005)** has reported the infection rate of *Theileria* in sheep in eastern Turkey to be 15.5%.

In China **(Milinget al., 2009;** and **Shuzhen et al., 2002)** recorded the incidence rate ranged from 10 to 15%.These results were nearly the same as the present work. On the other hand our result was higher than that detected by **Rjeibi et al. (2014)** who reported the first incidence of *T. lestoquardi* in small ruminants within the Maghreb region (1.2%). These variations might be regarded to the locations differences and sampling design as the present work was not focused on flocks with known theileriosis history but random selection were occurred.

The present results showed that no significance effect of season on the rate of infection (P>0.05). These results agreed with **Ahmed et al. (2003)** in Sudan as they observed that, incidence of *Theileria* infection did not vary much with season. The no significance effect of season might be attributed to continuous transmission of *Theileria* sp. in Egypt all over the year which support the fact that, the vector found to be active throughout most of the year even in small number in the study area.

Concerning age and sex susceptibility to infection present work showed no statistical significance between sex ; age and *Theileria* infection. **Razmi et al. 2003**; **and 2006** in Iran agreed with these findings. On other hand, **Bell-Sakyi et al. (2004)** in Ghana and **Dhaim and A'aiz (2014)** in Iraq showed that, *Theileria* infection did not affected with animal sex, while most of infection was showed among those animal with age equal or more than three years. This variation between present results and other studies may be attributed to difference in number of examined animals, immunological resistance, natural immunity and local climatic conditions which had effect on tick distribution.

By PCR *Theileria* was detected in 40% of examined cases while by microscopic examination it was 15.56%. PCR is more sensitive than microscopic examination. This result agreed with **Aktas et al. (2005)** who detected *Theileria* sp. in 15.5% of blood smears while 41.2% by PCR. **Yaghfoori et al. (2013)** observed *Theileria* spp. infection in Iran in 46% of blood smears, while 76 % were positive by using semi-nested PCR. In Bakestan, the Prevalence was 22% by microscopic examination and 35% by PCR (**Durrani et al. 2011).**

Molecular identification by PCR-RFLP technique, results demonstrated that, at least three genetically distinct *Theileria* spp. are found in Egypt RFLP proved presence of *T. ovis, T. lestoquardi* and *T. annulata* in Egyptian sheep. **Radwan and El Kelsh (2009)** found by molecular diagnosis that two species (*T. lestoquardi* and *T. ovis)* were proved to be the cause of ovine theileriosis in Egypt as well as another unknown species infecting sheep but with no clinical signs. RFLP proved to be effective and sensitive method for differentiation of *Theileria* sp. that infects sheep in Egypt without the need of highly expensive technique as sequencing, especially it is difficult to differentiate these species on the basis of morphology especially in mixed infections; this result agreed with other studies in different countries as mentioned by **Jalali et al. (2014)** and **HeidarpourBami et al. (2009)** in Iran and **Rjeibi et al. (2014)** in the Maghreb region.

From the above mentioned data we concluded that, it is necessary to develop a technique that is capable of detecting and discriminating all of the reported ovine *Theileria* species in Egypt without the need for sequencing which is very expensive and could not be applied on large scale. Exact identification of *Theileria* spp. helps in understanding its epidemiology then designing rational and cost-effective control strategies.

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