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DETECTION OF THEILERIA-ANNULATA CARRIER CATTLE IN EGYPT BY THE POLYMERASE CHAIN REACTION

(With 3 Tables and 2 Figures)

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الكشف عن الأبقار الحاملة للثابليريا النيولاتا في مصر بإستخدام تفاعل البلمرة المتسلسل

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أستخدمت في هذه الدراسة ستة وعشرون عينة دم – ستة عينات تم أخذهم من عجول معدية تجريبيا بطفيل الثابليريا النيولاتا والعشرون الأخرون من عجول سليمة ظاهريا من أربع محافظات بمصر معروفه مسبقاً أنها موبوءه بمرض الثابليريا. وقد تعرضت جميع العينات للفحص بالميكروسكوب العادي والميكروسكوب الفلورسنتي وأختبار البلمره المتسلسل ، وأسفرت النتائج عن وجود ١١عينه ايجابية من العشرين عينة بنسبة (٥٥٪) بالفحص الميكروسكوبي وعشرة عينات بنسبة (٥٠٪) بالميكروسكوب الفلورسنتي ، أما بالنسبة لإختبار البلمره المتسلسل فقد وجدت ١٦ عينه أيجابية بنسبة (٨٠٪) . ومن تلك النتائج يتضح أن إختبار البلمره المتسلسل ذو فائدة عالية كوسيلة لتشخيص الثابليريا النيولاتا في الأبقار الحاملة للطفيل حتى في حالة وجود الطفيل في الدم بمستوى منخفض.

SUMMARY

Twenty-six blood samples were used in this study. Six of them were collected from six experimentally infected calves with *T.annulata* strain while twenty blood samples were collected from asymptomatic cattle with theileriosis at four endemic provinces in Egypt. All samples were subjected to microscopic examination, fluorescent antibody test (IFA), and polymerase chain reaction (PCR). The carrier animals were detected in 11 out of 20

blood samples microscopically (55%) and in 10 of 20 by using IFA (50%) whereas PCR revealed that 16 samples were considered to be carrier positive(80%). Two sets of primers were used in PCR technique. A primer set A, derived from the sequence of the small subunit rRNA (SSUrRNA) of *Theileria* species, amplified 1.098 bp product for detection of *Theileria* spp. DNA. While a primer set B, deduced from the gene encoding the 30 KDa major merozoit surface antigen of *T. annulata* was successfully amplified 721 bp product. The PCR technique provides a useful diagnostic tool for detection of *T. annulata* carrier cattle even at low parasitaemia levels.

Key words: *Cattle - Theileria-annulata - PCR - Egypt*

INTRODUCTION

The tick-born protozoan parasite *Theileria-annulata (T.annulata)* is the causal agent of tropical theileriosis which is one of the most economically important diseases of grazing cattle (Williamson et al., 1989).

The disease threatens an estimating 250 million cattle world wide and acts as a major constraint on livestock production in many developing countries.

In Egypt, *T.annulata* infection affects cattle and buffaloes particularly in the endemic provinces such as El-Wadi El-Gedid, El-Fayoum and Gharbia (Hassanin, 1984 and El-Bahy, 1986). The percentage of *T. annulata* infection among cattle and buffaloes in Egypt was 59.36 % and 23% respectively (Rizkalla, 1991).

Control of bovine theileriosis in Egypt is primarily achieved by an eradication of tick vectors and the usage of different drugs for the treatment of the infected and carrier animals (Abdel-Rahman et al., 1987).
Such carriers are important causal factor to the infection within *Hyalomma* ticks which are able to transmit the infection to a susceptible host.

Detection of schizonts and piroplasms in Giemsa stained blood smears as well as the serological techniques such as the Indirect fluorescent antibody test (IFA) are the usual diagnostic methods for the detection of the parasite and the antibodies in the infected and carrier animals (Pipano and Cahana 1969). However, these methods are not capable of accurately diagnosing *T. annulata* carrier cattle even when the parasitaemia is at very low levels and it may be difficult to find the piroplasms in stained blood smears. Additionally, the antibodies tend to disappear in long-term carriers, whereas *Theileria* piroplasms persist (Dolan, 1986).

Recently, the polymerase chain reaction (PCR) which enzymatically amplifies specific DNA sequences *in vitro* (Mullis et al., 1986 and Saiki et al., 1988) has been successfully employed for the detection of different hemoparasites in cattle, including *Babesia bovis* (Fahrimal et al.,1992), *Theileria parva* (Bishop et al., 1992), *Theileria sergenti* (Tanaka et al., 1993) and *T.annulata* (D'Oliveira et al., 1995). The technique is sensitive and specific for the detection of even very low level of parasitaemia.

The aim of the present study is to use the PCR as a sensitive diagnostic technique for the detection of *T.annulata* carrier cattle in Egypt, in comparison with other conventional methods.

MATERIALS and METHODS

1- Experimental animals:

Six susceptible calves, one year old and weighing 250-300 Kg were used for the present study. They were proven to be free from *T. annulata* infection by using the conventional techniques as well as the PCR assay. The calves were experimentally inoculated by intravenous injection of 50 ml of infected blood with *T.annulata* according to Hawa (1977) and Pipano et al. (1977). Tracking of the experimental animals was executed by the examination of daily Giemsa stained blood smears and observation of lymph nodes appearance.

2- Test samples:

(a) - Blood samples from the experimentally infected calves :

Six blood samples were collected from experimentally infected calves with *T.annulata* at the parasitaemic peak (12 days post inoculation).

(b) - Blood samples from cattle under field condition :

Twenty blood samples were collected from cattle with no symptoms of theileriosis at four geographically different provinces in Egypt which previously proved to be endemic areas of theileriosis. The four provinces were : El-Wadi El-Gedid, El-Fayoum, Tanta (El-Gharbia), and Wadi El-Natroon (El-Bahera). All blood samples were randomly collected as two milliliters aliquots on EDTA and stored at - 20^0C until used for PCR amplification. Giemsa stained blood smears were prepared for the microscopic examination of the parasites, and serum samples were collected for Indirect fluorescent antibody technique (IFA).

3- Microscopic examination

Thin blood smears stained with Giemsa stain were prepared from the ear vein of all animals and examined for the presence of *T.annulata* parasites (Schalm et al., 1975).

4- Indirect fluorescent antibody test (IFA) :

The technique was performed on the serum samples collected from all the blood samples according to Pipano and Cahana (1969). Rabbit antibovine IgG fluorescein isothiocyanate (FITC) conjugate was used and supplied by Sigma Chemical Company, Saint Louis, USA.

5- Polymerase chain reaction (PCR) :

a- Processing samples for PCR

All the blood samples were processed as described by Barker et al. (1992). Briefly, 500 ul of saponin lysis mixture (0.22% Nacl, 0.015% Saponin, and 1mM EDTA) were added to 100 ul of blood sample and the mixture was centrifuged at 14000 r.p.m. for 1 min. Pellets were washed three times with 0.5 ml of saponin lysis mixture, resuspended in 150 ul of PCR buffer (50 mM Kcl, 10 mM Tris-Hcl [pH 8], 0.5% Tween 20, and 100 ug of proteinase K / ml) and were incubated at 56^0C for 1h. After 10 min. of incubation at 95^0C, 5 ul was taken for PCR.

b- Polymerase chain reaction (PCR) amplification

Two sets of primers were used and mentioned in Table (1) : primer set [A] : No. 989, 990 was used for amplification of *Theileria* species DNA and primer set [B] : N/516, N/517 was used for amplification of *T.annulata* DNA. PCR amplification was performed, as described by D'Oliveira et al. (1995) with some modification, in a total reaction of 50 ul :

1-For primer set A : The reaction containing 50 mM Kcl, 10 mM Tris-Hcl (pH 8.3),4 mM $MgCl_2$, 0.1% Triton X 100, 200 uM of the deoxynucleotide triphosphate (dNTPs) mix (Gibco BRL, USA), 2 units of Taq polymerase enzyme (Promega Company, Madison, USA), 80 Pmole of primer set A, and 5 ul of processed blood samples.

2- For primer set B : The reaction containing 50 mM Kcl, 10 mM Tris-Hcl (pH 8.3), 1.5 mM $MgCl_2$, 0.1% Triton X 100, 200 uM of dNTPs mix ***(Gibco BRL, USA)***, 2 units of Taq polymerase enzyme (Promega Company, Madison, USA), 80 Pmole of primer set B, and 5 ul of processed blood samples.

The negative control samples for primer set A & B contained all the reaction components without template. The reaction was overlaid by light mineral oil.

The reaction proceeded in automatic DNA thermal cycler (Robocycler, Stratagene company, La Jolla, CA, USA) with a three steps cycling program as follows : 35 cycles :denaturing at 94^0C for 1 min,. for ***primer set A*** annealing at 55^0C for 1 min. While for ***primer set B***

annealing at 52°C for 1 min. and, extension at 72°C for 1.5 min., and 1 cycle: extension at 72°C for 10 minutes.

Table 1:

Oligonucleotide primers used to amplify 30-KDa and SSUrRNA genes sequence of *T.annulata*.

Primer name	Primer sequence	Position
**Primer set A*		
989 (forward primer)	5'- AGTTTCTGACCTATCAG-'3	278-294
990 (Reverse primer)	5'-TTGCCTTAAACTTCCTTG-3	1376-1359
***Primer set B*		
N516 (forward primer)	5' -GTAACCTTTAAAAACGT-'3	243-250
N 517(Reverse primer)	5' GTTACGAACATGGGTTT-'3	954-938

*, ** = were synthesized by ***(Gibco-BRL, USA).***

C- Analysis of amplified PCR products

The amplified PCR products were analyzed by 1% agarose gel electrophoresis and stained with ethidium bromide (0.5 ug/ml) for 30 min. using a standard DNA molecular marker ***(1Kb DNA ladder, Gibco-BRL, USA).***

RESULTS

The obtained results are illustrated in Tables 2 & 3 and Fig. 1&2).

DISCUSSION

The control of theileriosis in the carrier animals represents a serious problem since they are important contributors to the infection within *Hyalomma* ticks. The low levels of infection with *T.annulata* can maintain a tick transmissible carrier state in asymptomatic cattle.

Although *T. annulata* infection can be detected routinely by conventional microscopic examination, it lacks sensitivity and inconsistency of results. Using the IFA test for antibody detection, the technique shows cross - reactivity with other *Theileria* species and can not always detect the long-term carriers (Burridge et al., 1974 and Kiltz et al., 1986).

The high sensitivity of the synthetic oligonucleotide set A, derived from SSUrRNA gene in PCR amplification for the detection of *Theileria* species DNA, was shown in Table (2). The results of the collected blood

samples from the experimentally infected calves with *T. annulata* were positive using PCR technique, thus confirming the positivity of microscopic examination and IFA test. The positivity of the PCR product is clear in Fig. (1) where the expected DNA fragment was detected at 1,098 bp. These results are supported by D'Oliveira et al., (1995) who used Theileria species specific primer (989 and 990), deduced from small subunit (SSUrRNA) gene for amplification of the expected 1,098 bp DNA fragment from all Theileria species samples examined.

Since the primer set A can not differentiate among all members of Theileria species and the most predominant strain in Egypt is *T.annulata* (Liebisch et al., 1984 and Abd El-Rahman et al., 1989), so we have focused on the usage of specific primer derived from the gene encoding 30 - KDa major merozoite antigen (primer set B) for specific amplification of *T.annulata* DNA (Dickson and Shiels, 1993 and D'Oliveira et al., 1995).

The results of PCR amplification of experimentally infected blood samples were clarified in Table (2) and Fig. (2). All the blood samples collected from the experimentally infected calves and previously proved to be positive with primer set A, gave the expected 721 bp amplified PCR product with the primer set B. The obtained result indicated that the amplified DNA fragment was specific for *T. annulata*. This finding comes in agreement with the result obtained by D'Oliveira et al. (1995) who successfully amplified four stocks of *T.annulata* DNA from distant geographic regions indicating that a target gene sequence (30-KDa major merozoite surface antigen) is conserved with *T.annulata*.

Amplification of parasite DNA by PCR is far more sensitive than parasite detection using light microscopy or IFA test as demonstrated with field samples in Table (3).The carrier animals were detected in only 11 out of 20 (55%) samples by microscopic examination and in 10 samples (50%) by IFA test whereas 16 samples were considered to be carrier using the PCR assay (80%).

It is interesting to mention that the PCR system able to detect the parasites even with a low parasitaemia. This phenomenon was obvious, particularly, in two blood samples No. 6 and 7 (Table 3), where the parasitaemia was at low level (o.1% and 0.2% respectively). Our result comes in harmony with Bishop et al. (1992) who reported that the PCR using *T.parva*-specific primers is capable for detecting *T.parva* parasites at low parasitaemia level in carrier cattle. Similarly, Fahrimal et al. (1992), Tanaka et al. (1993) and Kawazu et al. (1995) stated that PCR is

significantly more sensitive than the current methods since it can detect even the low level of parasites in the blood.

Also, worth considering from the results illustrated in Table (3) is that 3 blood samples No. 9, 10, and 11 confirmed to be infected with *T.annulata* by the light microscopy and PCR reaction, while they gave negative results using IFA test. This false-negative IFA result could be attributed to the fact that antibodies tend to disappear in long term carriers, whereas Theileria piroplasms persist. Therefore, animals with negative serological test (IFA) can still infect ticks (Dolan, 1986 and Bishop et al., 1992).

It is important to point out that amplification of parasite DNA using the PCR assay has advantages over the light microscopy as shown by the fact that blood samples No.12 and 13 were demonstrated positive by PCR amplification while gave negative result by microscopic examination (Table 3). This important fact was explained by Bishop et al. (1992) and Russomando et al. (1992) who stated that during the chronic stages (carrier), the low parasitaemia often precludes the detection of the parasite in the blood samples.

Since microscopic examination and IFA techniques gave false negative results and failed to detect *T.annulata* piroplasms and antibodies in four blood samples No. 14, 15, 16, and 17, the PCR, on the other hand, gave positive results. This striking finding proved that PCR amplification is more efficient and superior to existing methods.

The non detectable PCR amplification in blood samples No. 18, 19, and 20 came concurrently with the negative results given by microscopic examination and IFA test. Thus it is likely that these blood samples were free from *T.annulata* parasite.

Importantly, it is worth mention that one blood sample No.8 was found to be positive by microscopic examination and IFA test but negative by PCR. This result could be accounted for the presence of residual inhibitory components in the blood sample including intracellular substances such as the porphyrin ring of heme, which is thought to bind Taq I polymerase enzyme and consequently inhibit the PCR amplification (Higuchi, 1989).

In conclusion, the results obtained during this study suggest that the PCR system using *T.annulata* specific primer is highly sensitive and specific technique being used as an excellent confirmatory test with microscopic examination and IFA test particularly when the conventional methods

gave negative results with *T.annulata* in endemic areas. Additionally, the method can be used to assess parasite clearance from the blood, thus offers an alternative approach for the detection of the carrier status of *T.annulata* infected cattle under field conditions.

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Table (2): The results of different techniques (microscopic examination, fluorescent antibody test, polymerase chain reaction) for the detection of experimentally infected cattle with *T.annulata*.

Sample No.	Microscopic examination		Indirect fluorescent antibody test (IFA)	PCR for *Theileria* Species	PCR for *Theileria annulata*
	% of parasitaemia	Result			
1	12	+	+	+	+
2	11	+	+	+	+
3	12	+	+	+	+
4	10	+	+	+	+
5	8	+	+	+	+
6	6	+	+	+	+

+ = positive result

Table (3): The results of microscopic examination, fluorescent antibody test, and polymerase chain reaction for the detection of *T.annulata* in cattle blood samples under field condition.

Sample No.	Microscopic examination		Indirect fluorescent antibody test (IFA)	PCR for *Theileria* Species	PCR for *Theileria annulata*
	% of parasitaemia	Result			
1	7	+	+	+	+
2	7	+	+	+	+
3	6	+	+	+	+
4	3	+	+	+	+
5	2	+	+	+	+
6	0.2	+	+	+	+
7	0.1	+	+	+	+
8	1	+	+	-	-
9	6	+	-	+	+
10	6	+	-	+	+
11	5	+	-	+	+
12	-	-	+	+	+
13	-	-	+	+	+
14	-	-	-	+	+
15	-	-	-	+	+
16	-	-	-	+	+
17	-	-	-	+	+
18	-	-	-	-	-
19	-	-	-	-	-
20	-	-	-	-	-

+ = positive result - = Negative result

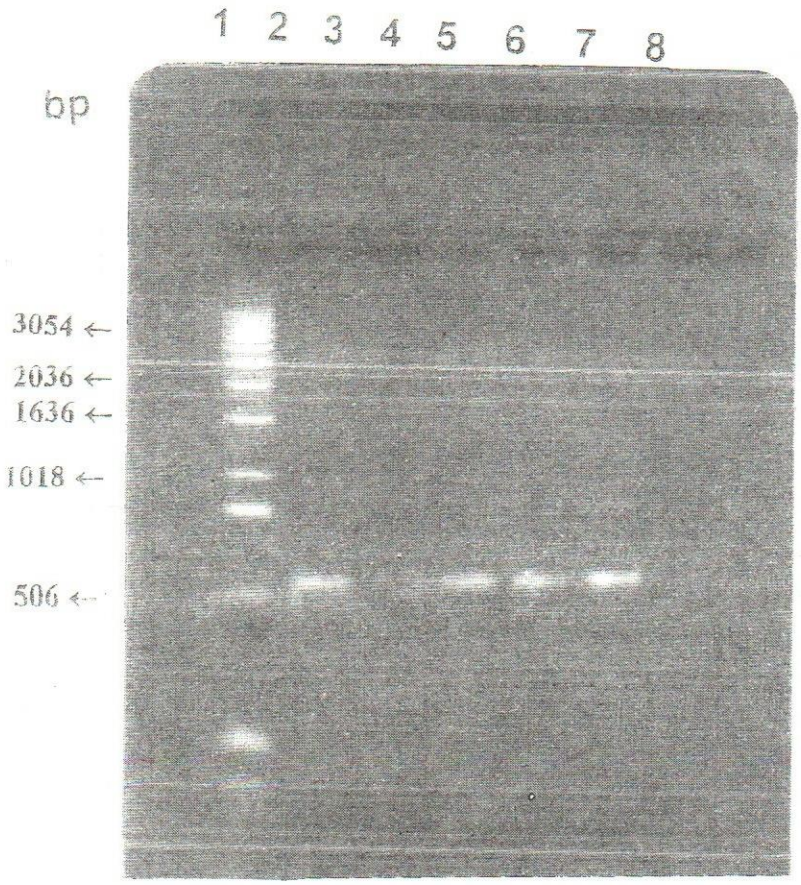

Fig.(1) : Agarose gel electrophoresis of amplified *Theileria* species DNA (1,098 bp PCR product) by using primer set A. ***Lane (1)*** :1Kb DNA ladder as a standard marker, ***Lane (2)*** : positive control PCR product amplified from blood sample collected from experimentally infected calf with *T.annulata* (sample No.3), ***Lane (3)*** : Negative PCR product (sample No.18), ***Lane (4-6)*** : Positive PCR product amplified from field blood samples (No.3, 9, and12), and Lane(7) : Negative control(No amplified PCR product).

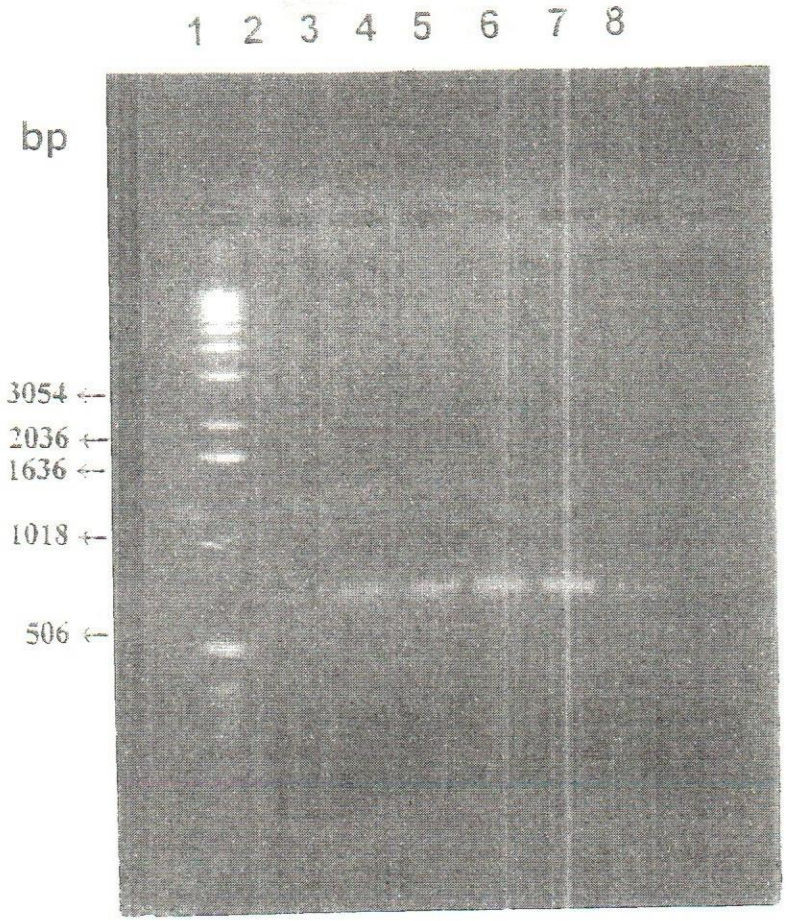

Fig. (2) : Agarose gel electrophoresis of amplified *T.annulata* DNA(721 bp PCR product) by using primer set B. ***Lane (1)*** :1Kb DNA ladder as a standard marker. ***Lane (2-5)*** : positive PCR product amplified from field blood samples (No.3,7, 9. and12)., ***Lane (6-7)*** : positive control PCR product amplified from blood sample collected from experimentally infected calf with *T.annulata* (sample No.3 & 4 respectively), and ***Lane(8)***: Negative control (No amplified PCR product).