DIAGNOSIS OF THEILERIOSIS IN CATTLE BY USING POLYMERASE CHAIN REACTION (PCR)

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ABSTRACT

The study evaluated the usefulness of specific PCR assays for the diagnosis of tropical theileriosis caused by Theileria annulata in Egypt using Microscopic Examination (ME) for comparison. Blood samples from 70 animals comprising both diseased and apparently healthy cattle were examined for the presence of Theileria infection by ME using Giemsa-stained blood smears, where the prevalence was 17.1% (12/70). To evaluate the usefulness of PCR assays for the identification of Theileria sp. and T. annulata, thirty bovine blood samples selected from the seventy animals were tested by both PCR assays and ME. Two primer were used: first primer based on small subunit (SSU rRNA) gene for common Theileria species were used to demonstrate the presence of Theileria DNA and second specific primers derived from the gene encoding 30 KDa major merozoite antigen to specifically amplify T. annulata DNA in the examined bovine samples. I identified 7/30 (23.3%), whereas the PCR assay for Theileria sp. was more sensitive which identified 25/30 (83.3%). Of these 25 Theileria positive samples, 22 were identified as infected with T. annulata by specific PCR assay for T. annulata. This study demonstrated that, PCR methods are more sensitive and accurate for diagnosis of tropical theileriosis than the common method (ME).

INTRODUCTION

Theileria annulata is a tick-borne protozoan parasite of cattle, which is also known as Mediterranean or Tropical Theileriosis has vast geographical spread and can be found especially in Middle East, Mediterranean Europe, North of Africa, India, Middle Asia and even China **(D'oliveira et al., 1995)**. The disease threatens an estimated 250 million cattle worldwide and acts as a major constraint on livestock production in many developing countries.

Ticks of the genus Hyalomma are the main vector for T. annulata **(Robinson, 1982)**. If the animal recovers from infection, long-lasting carrier status occurs in which low numbers of erythrocytes remain infected with T. annulata piroplasms. These carrier animals have an important role in the transmission of infection by the Hyalomma ticks **(D'Oliveira et al., 1995)**. Other species of Theileria with low pathogenicity or non-virulence also exist, which under field conditions needs to be distinguished from the pathogenic T. annulata.

Classical laboratory diagnosis of theileriosis is based on Microscopic Examination (ME) of the parasite in thin smears of blood and on the presence of macroschizonts in Giemsa-stained lymph node biopsy smears. However, ME detection of piroplasms has low sensitivity due to low number of parasites in carrier cattle leading to high false negative diagnosis. In addition, it does not allow the differentiation of Theileria species causing the infection, which differ in their pathogenicity. Serological tests such as the Indirect Fluorescent Antibody Technique (IFAT) and Enzyme-Linked Immunosorbent Assay (ELISA) also have some disadvantages. Antibodies tend to disappear in long-term carrier cattle despite the presence of piroplasms and furthermore, cross-reactivity with antibodies directed against other species limits the specificity of IFAT (**Burridge et al., 1974; Papadopoulos et al., 1996**). Moreover, ELISA lacks either sensitivity or specificity. False positive and negative results are commonly observed in serological tests due to cross-reaction, weakening in specific immune response as well as lack of determination of antibodies in carriers because of long term infection (**Leemans et al., 1999; Gubbels et al., 2000**).

Polymerase Chain Reaction (PCR) assays have been successfully employed for the detection of different haemoparasites in cattle including Babesia bovis (**Fahrimal et al., 1992; Calder et al., 1996**), Theileria parva (**Bishop et al., 1992**), Theileria sergenti (**Tanaka et al., 1993**), Babesia bigemina (**Figueroa et al., 1992; Salem et al., 1999**) and T. annulata (**D'Oliveira et al., 1995**). **Kirvar et al. (2000)** reported that, the sensitivity of this method is diagnosing one parasite per ml of blood and its accuracy of being three times more than detection of parasite by smear method microscopically. **D'oliveira et al. (1995)** demonstrated that, the lowest extent of PCR T. annulata carrier diagnosing 2-3 parasites in blood unit and that amount is equal to 0.000048% of parasitemia.

The objective of the present study was to evaluate PCR assay for sensitive and specific amplification of Theileria sp. and T. annulata against standard test (ME) from blood samples obtained from both apparently healthy and diseased cattle in El-Dakahlia and Kafr El-Sheikh provinces in Egypt.

MATERIALS AND METHODS

Animals and area of study :

This study was conducted on 70 cows of different ages and breeds in El-Dakahlia and Kafr El-Sheikh provinces representing the East Delta region in Egypt. Of these, 43 cross breed cows were sampled from EL-badrawy farm at Nabroh city, El-Dakahlia province and 27 cross breed cows were sampled from El-Kasaby farm at Bialla city, Kafr El-Sheikh province.

Some of the examined animals were suffering from fever, lacrimation, conjunctivitis, enlarged superficial lymph nodes and anemia in addition to the presence of ticks on different parts of the animal's body. Others were apparently normal but examined carefully both clinically and parasitologically to detect the subclinical cases.

Sampling :

Two blood smears from the ear vein of each examined animal were collected after clipping, sheaving and disinfection of the intended area for detection of Theileria piroplasms microscopically after staining with Giemsa stain. Moreover, Approximately 10 ml blood samples were collected from Jugular vein on tubes containing EDTA and stored at -20°C for DNA extraction. The blood samples used for PCR evaluation were obtained from 30 selected animals to represent different ages, species, breeds, sexes and clinical symptoms from the animals of the studied farms.

Microscopic Examination (ME) :

Blood films were prepared as previously described **Kelly, (1979)** by picking the ear with a sharp needle after the ear was clipped clean by alcohol and dried. Immediately, a small drop of fresh blood was stocked to clean slide then, rapidly spread into an even thin film by a second clean slide held at 45° angle and immediately dried. The slides were labeled and kept in upright position in a special box and carried to the laboratory. The blood films were quickly fixed in methyl alcohol (99%) for 5 minuts and stained with 5% Giemsa stain solution for 30 min and examined with an oil immersion lens at a total magnification of x 1,000 for the presence of Theileria piroplasms. Each blood film was examined three times before being considered negative.

DNA extraction and PCR amplification :

Extraction of Theileria genomic DNA from 200µl of EDTA-treated whole blood was performed according to the method described by **Holman et al., (2000 and 2002).** Positive control samples representing T. annulata and Theileria sp. were obtained from clinical cases diagnosed at the Faculty of Veterinary Medicine, Mansoura University, Egypt. All the DNA samples were stored at -20°C until further use.

PCR reactions were performed in a total volume of 50µL, containing PCR buffer (50mM KCl, 10mM Tris-HCl, 4mM MgCl2), 200µM of each dNTP, 80pmol of each primer and 2U of Taq DNA polymerase enzyme (Promega). The primers used for amplifying Theileria sp. are described by **D'Oliveira et al. (1995)**, while the specific primers to T. annulata were synthesized as described by **Allsopp et al. (1994)** (Table 1).

Amplification was performed in a thermocycler (Coy Corporation) under the following conditions, 95°C for 5 min (initial denaturation) followed by 35 cycles of 94°C for 1 min (denaturation), 55°C for 1 min (annealing), 72°C for 1.5 min. (extension), and a final extension at 72°C for 10 min.

Five µl of genomic DNA were added to each PCR reaction. Positive control and negative control (with no DNA template) were always included in each PCR amplification. The amplification products were subjected to electrophoresis on 1.5% agarose gel containing ethidium bromide. The length of the amplified products was estimated by using a molecular weight marker (100 bp ladder, Amersham Biosciences) and the amplified products were visualized with an UV transilluminator and gel photographs were taken by a digital camera.

Table 1: Primers used for PCR amplification of *T. annulata* and *Theileria sp.*

Parasite	Primers	Sequence (5' – 3')	Product size
T. annulata	Forward	GTAACCTTTAAAAACGT	721 bp
T. annulata	Reverse	GTTACGAACATGGGTTT	
Theileria sp.	Forward	AGTTTCTGACCTATCAG	1098 bp
Theileria sp.	Reverse	TTGCCTTAAACTTCCTTG	

RESULTS AND DISCUSSION

Out of the 70 animals examined by ME, 12 (17.1%) were found to be infected with Theileria. While in the other 58 samples, these piroplasmic forms could not be found, so that they were considered negative. PCR reaction was performed on extracted DNA from 30 blood samples and amplified products were separated by electrophoresis on a 1.5% agarose gel, then the results were analyzed. The amplified sequence for Theileria sp. and T. anuulata were 1098 and 721, respectively. The size of PCR products was determined by a molecular weight marker that was run in parallel to the PCR products. Thirty bovine blood samples representing different breeds, ages, and localities were used to evaluate the efficiency of specific PCR assays using ME for comparison. The results showed that the ME identified 7/30 (23.3%), whereas the PCR assay for Theileria was more sensitive, which identified 25/30 (83.3%; Fig. 1). Of these 25 positive Theileria samples, 22 (88%) were diagnosed to be infected with T. annulata (Fig. 2).

Cattle with subclinical theileriosis become chronic carriers of the piroplasm and hence, the sources of infection for tick vectors. Therefore, latent infections are important in the epidemiology of the disease. The diagnosis of piroplasm infections is based on clinical findings and microscopic examination of Giemsa-stained blood smears. However, this method is not sensitive enough or sufficiently specific to detect chronic carriers, particularly when mixed infections occur. For this reason, PCR assays have been the most preferred method for detection of Tick Borne Diseases (TBD). Several studies have documented that PCR assays are more sensitive and specific than conventional diagnostic techniques in determining piroplasm-carrier animals **(D'Oliveira et al., 1995; Aktas et al., 2002; Altay et al., 2007)**.

D`oliveira et al., (1995) contaminated 92 one-year-old cattle of Bostaurus breed with four strains of T. annulata which were collected from Turkey, Spain, Portugal, and Mauritania and tried to diagnose this experimental contamination by PCR, IFA and smear. The positive cases in smear, IFA and PCR methods were 22, 40 and 70%, respectively. Moreover, **Roy et al., (2000)** reported that in detection of 50 blood samples of native cows by PCR and smear methods, the positive cases by PCR were 42 and by Smear method only 8 of them were positive. In addition, **Martin-**

Sanches et al., (1999) showed that PCR method had more accuracy and sensitivity in comparison with the smear method. **Mahmmod et al., (2010)** evaluated the usefulness of specific PCR assays for the diagnosis of tropical theileriosis caused by T. annulata in Egypt using ME for comparison. He showed that PCR assay for Theileria sp. was more sensitive than ME.

In this study, the conserved PCR primers based on small subunit (SSU rRNA) gene for common Theileria species were used to demonstrate the presence of Theileria DNA (Fig. 1) in the examined bovine samples. But this pair of primers fails to differentiate among members of Theileria species, demonstrating that, the most predominant Theileria species in Egypt is T. annulata, so we then used the specific primers derived from the gene encoding 30KDa major merozoite antigen to specifically amplify T. annulata DNA **(Dickson and Shiels, 1993; D'Oliveira et al., 1995)**.

It is important to point out that PCR assay has advantages over ME, because some bovine blood samples give negative results by ME but were positive by PCR amplification. The results of this study also suggested that, Theileria sp. rather than T. annulata are also present in Egypt because some samples gave positive results by PCR for Theileria sp. but did not amplify with T. annulata. These could be non-pathogenic Theileria species such as T. mutans, T. buffeli or T. orientai.

CONCLUSION

The results demonstrated that, the used PCR assay detects T. annulata at low parasitemias in carrier cattle. The ability of the present PCR assay to discriminate T. annulata from nonpathogenic Theileria species suggests its use as a routine technique for diagnosis of tropical theileriosis.

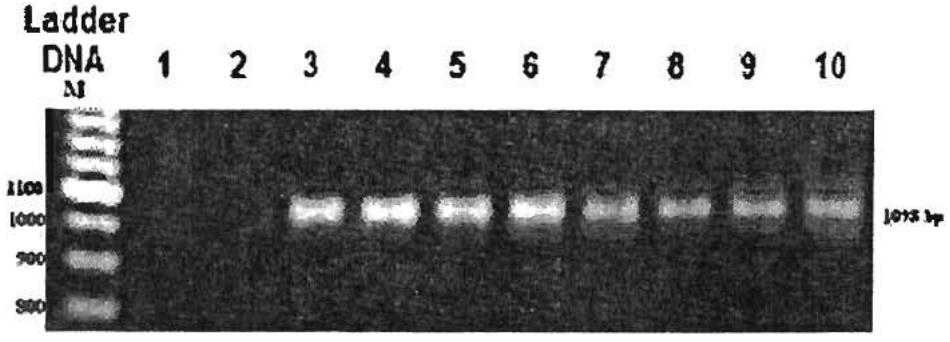

Fig.(1) : Agarose gel electrophoresis of amplified *Theileria* sp. DNA by using PCR and primers for *Theileria* sp.

Lanes 3-10 show 1098 bp PCR products from field bovine blood samples, lanes 1-2 are negative controls from healthy cows, and lane M represents a 1 Kb DNA ladder. The size of the marker (in bp) is shown on the left of the image.

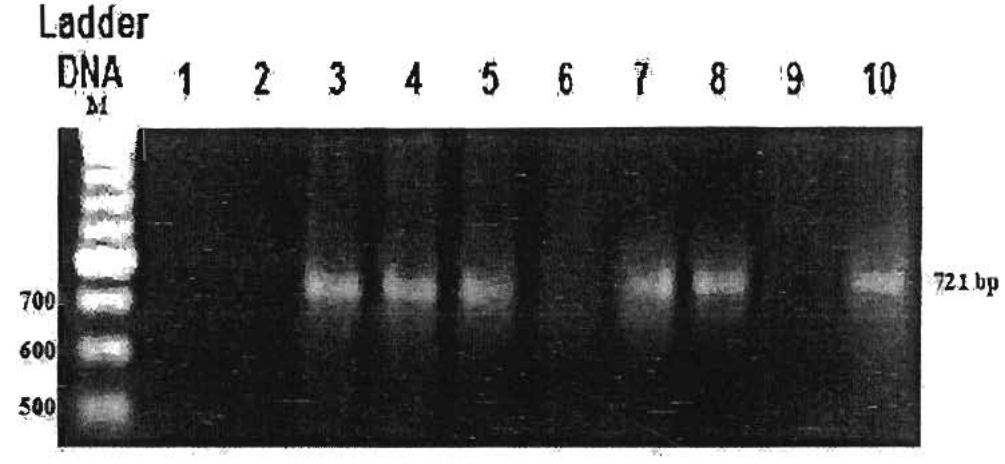

Fig. (2): Agarose gel electrophoresis of amplified *T. annulata* DNA by using PCR and specific primers to *T. annulata*.

Lanes 3, 4, 5, 7, 8, and 10 show 721 bp PCR products from field bovine blood samples, lanes 6, 9 represent negative samples for *T. annulata*, and lane M represents 1 Kb DNA ladder.

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الملخص العربى

تفاعـل البلمـرة المتسلسل في تشخيص الثيليريـا في الأبقـار

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أجريت هذه الدراسة لتقييم فاعلية تفاعل البلمرة المتسلسل المتخصص لتشخيص الثيلريا الإستوائية في الأبقار و التى يسببها طفيل الثيلريا فى مصر بالمقارنة مع الفحص الميكروسكوبى . اعتمد البحث على تجميع عدد ٧٠ عينة دم من الحيونات المصابة و أخرى سليمة ظاهريا وتم فحص عينات الدم بواسطة الفحص الميكروسكوبى بإستخدام صبغة الجيمسا .

في هذه الدراسه ,تم إستخدام زوج من البرادئ لفحص طفيل الثيلريا وزوج أخر من البرادئ متخصص لفحص طفيل الثيلريا الإستوائية فى عدد ٣٠ عينة دم بالاضافه الى الفحص الميكروسكوبى لهذه العينات.

اوضحت النتائج ان نسبة الاصابه بالمرض باستخدام الفحص الميكروسكوبي كانت ٢٣٫٣٪ (٧/ ٣٠) بينما كانت نسبه الاصابه ٨٣٫٣٪ (٢٥/ ٣٠) باستخدام تفاعل البلمرة المتسلسل.

من هذه النتائج يتضح ان استخدام تفاعل البلمرة المتسلسل لتشخيص الثيلريا الإستوائية في الأبقار يعتبر أكثر حساسية ودقه عن الفحص الميكروسكوبى.