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Field Evaluation of PCR Assays for the Diagnosis of Tropical Theileriosis in Cattle and Water Buffaloes in Egypt

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Abstract: The study evaluated the usefulness of specific PCR assays for the diagnosis of tropicaltheileriosis caused by *Theileria annulata* in Egypt using Microscopic Examination (ME) for comparison. Blood samples from 258 animals comprising both sick and apparently healthy cattle and water buffaloes were examined for the presence of *Theileria* infection by ME using Giemsa-stained blood smears and the prevalence was 18.6% (48/258). To evaluate the usefulness of PCR assays for the identification *Theileria* sp. and *T. annulata*, 30 bovine blood samples selected from the 258 animals were tested by both PCR assays and ME and ME identified 9/30 (30%), whereas the PCR assay for *Theileria* sp. was more sensitive which identified 21/30 (70%). Of these 21 *Theileria* positive samples, 15 were identified as infected with *T. annulata* by specific PCR assay for *T. annulata*. This study demonstrated that specific PCR assays are more sensitive and accurate for the clinical diagnosis of tropical theileriosis.

Key words: Cattle, diagnosis, microscopic examination, PCR, tropical theileriosis, *Theileria annulata*, water buffaloes, Egypt

INTRODUCTION

Theileria annulata, a tick-borne protozoan parasite of cattle and domestic buffalo (*Bubalus bubalis*) is the causative agent of tropical theileriosis. This disease also called Mediterranean theileriosis in the Mediterranean basin is one of the most fatal types of theileriosis in Europe, North Africa and Asia extending through the Middle East, India and Southern Russia into China (Williamson *et al*., 1989). The disease threatens an estimated 250 million cattle worldwide and acts as a major constraint on livestock production in many developing countries.

Tropical theileriosis affects cattle and buffaloes in El-Wady El-Gedid province, Egypt at in a percentage 59.36 and 23%, respectively. 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 numbers of parasites in carrier cattle leading to high false negative diagnosis. Also, 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 disadvantages. Antibodies tend to disappear in

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long-term carrier cattle despite the presence of piroplasms and further more, cross-reactivity with antibodies directed against other species limits the specificity of IFAT (Burridge *et al*., 1974; Papadopoulos *et al*., 1996). ELISA lacks either sensitivity or specificity.

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). The objective of the study was to evaluate PCR assay for sensitive and specific amplification of *Theileria* sp. and *T. annulata* against Gold standard test (ME) from blood samples obtained from both apparently healthy (carrier) cattle and buffaloes and diseased ones in the Delta region in Egypt.

MATERIALS AND METHODS

Examined animals and area of study: This study was conducted on 258 animals (96 buffaloes and 162 cows) of different ages, breeds from Sharkia, El-Dekhlia and Ismalia provinces representing the East Delta region in Egypt. Of these, 195 buffaloes and cows were sampled from the Veterinary Clinic of Zagazig Veterinary Medicine Faculty and 17 Brownswiss breed cows from Sekam farm at Billbis city, Sharkia Province, 37 crossed breed cows were sampled from El-Bieomy farm at Gamasa city, El-Dekhlia Province, 9 crossed breed cows were sampled from Marrwad farm at El-Salhia El-Gededa city, Ismalia Province.

Some of the examined animals were suffering from fever, lacrimation, conjunctivitis, enlargement of superficial lymph nodes and anemia in addition to the presence of ticks on different parts of the animal body. Others were apparently normal but examined carefully both clinically and parasitologically (Kelly, 1979) 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. 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 and Veterinary Clinics.

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

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

PCR reactions were performed in a volume of 50 μL, containing PCR buffer (50 mM KCl, 10 mM Tris-HCl, 4mM $MgCl_2$), 200 μM of each dNTPs, 80 pmol of each primer (Table1) and 2 U of *Taq* DNA polymerase enzyme (Promega). The conserved primers for amplifying *Theileria* sp. used those described by D'Oliveira *et al*. (1995), while the primers specific 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, 1 min (denaturation), 55°C, 1 min (annealing), 72°C, 1.5 min (extension) and a final extension of 72°C for

Table 1: Primers for the amplification of *Theileria* sp. and *Theileria annulata* used in the study

Parasites	Primers	Primer sequence (from 5'-3')	Product size	References
Theileria sp.	F (989)	AGTTTCTGACCTATCAG	1098 bp	D'Oliveira *et al*. (1995)
Theileria sp.	R (990)	TTGCCTTAAACTTCCTTG		
T. annulata	F (N516)	GTAACCTTTAAAAACGT	721 bp	Allsopp *et al*. (1994)
T. annulata	R (N517)	GTTACGAACATGGGTTT		

F: Forward primer; R: Reverse primer

10 min. Five microlitres of genomic DNA was 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 base pair standard (100 base-pair ladder, Amersham Biosciences) and the amplified products were visualized with an UV transilluminator (Sigma).

RESULTS AND DISCUSSION

Out of the 258 animals examined by ME, 48 (18.6%) were found to be infected with *Theileria*. The infection rate for theileriosis in Zagazig Veterinary Clinic was 22.6% (44/195) and was 2.7% (1/37) in El-Biomy Farm, 17.6% (3/17) in Sekam Farm and 0% (0/9) in Marrwad Farm. The difference in infection rate may explain the level of management including the tick control programs which varied from herd to herd and from farm to farm in the same locality.

Thirty bovine blood samples representing different species, 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 9/30 (30%), whereas the PCR assay for *Theileria* was more sensitive which identified 21/30 (70%) (Fig. 1). Of these 21 positive *Theileria* samples, 15 (71.4%) 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 diseases. 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).

In this study, the conserved PCR primers based on small subunit (SSU rRNA) gene for common *Theileria* species (D'Oliveira *et al*., 1995) (Table 1) were used to demonstrate the presence of *Theileria* DNA (Fig. 1) in the examined bovine samples. But this pair of primers can not differentiate among members of *Theileria* species. Given that the most predominant *Theileria* species in Egypt is

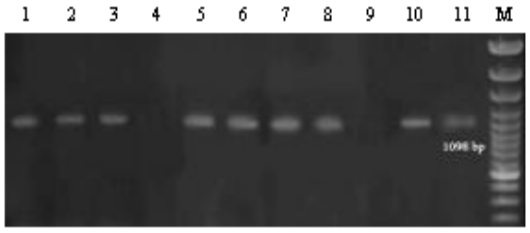

Fig. 1: Agarose gel electrophoresis of amplified *Theileria* sp. DNA (1098 bp PCR product) using primers common to *Theileria* sp. Lanes 1, 2, 3, 5, 6, 7, 8, 10 represent positive amplifications from field bovine blood samples, lanes 4 and 9 represent negative amplifications from field blood samples, lane 11 represents positive control sample and lane M represents 1 Kb DNA ladder as a standard size marker

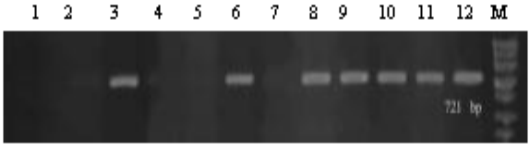

Fig. 2: Agarose gel electrophoresis of amplified *Theileria annulata* DNA (721 bp PCR product) by using primers specific to *T. annulata*. Lanes 3, 6, 8, 9, 10, 11 represent positive amplifications from field bovine blood samples, lanes 1, 2, 4, 5, 7 represent negative amplifications from field blood samples, lane 12 represents positive control sample and lane M represents 1 Kb DNA ladder as a standard size marker

T. annulata, so we then used the specific primers derived from the gene encoding 30-KDa 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 the study also suggest 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. oriental*, which warrants further studies.

CONCLUSION

The results demonstrate that this 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.

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