

DIAGNOSTIC AND GENETIC STUDIES OF *THEILERIA ANNULATA* WITH SPECIAL REFERENCE TO GENETIC POLYMORPHISM OF *THEILERIA ANNULATA* MEROZOITE SURFACE (TAMS-1) ANTIGEN

AMIRA A.T. AL-HOSARY* ; LAILA S. AHMED* and ULRIKE SEITZER**

* Department of Animal Medicine (Infectious diseases), Faculty of Veterinary Medicine, Assiut University, Assiut, Egypt, PO Box 71526.

** Division of Veterinary Infection Biology and Immunology, Research Center Borstel, parkallee 22, 23845, Borstel, Germany.

Email: amiraelhosary@yahoo.com

ABSTRACT

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Diagnosis of bovine theileriosis was carried out by blood film and polymerase chain reaction-PCR methods. In the current study, *Theileria annulata* merozoite piroplasm Surface Antigen (Tams-1) target based PCR was used for specific detection of *T. annulata* infection in cattle from different localities in Upper Egypt then followed by cloning and sequencing of this gene then alignment of all obtained sequences and their translated amino acids to studying its polymorphism among different local strains. The results of this study concluded that blood film is still important for diagnosis. (Tams-1) target based PCR test was more sensitive than blood film. The infection rates with *T. annulata* in the examined cattle were 46.19% and 28.57% by using Tams-1 target based PCR and thin blood film, respectively. The (Tams-1) gene sequencing, alignment and translation into amino acids concluded that Tams-1 sequences and its translated amino acids were highly variable. This makes it not recommended for use in vaccination and/or serological diagnostic tests. The sequences and their translated amino acids were deposited in the GenBank™ databases and available under accession numbers as following (GenBank™: KJ021626, GenBank™: KJ021627, GenBank™: KJ021628, GenBank™: KJ021629).

Keywords: *Diagnosis; Blood film, PCR, Tams-1; Theileria annulata.*

INTRODUCTION

Theileriosis is the most economically important disease of cattle; it causes severe losses in livestock production in Egypt Al-Gaabary, (1995) and AL-Hosary, (2009). (Tams-1) is an antigen of *T. annulata*, it is one of the merozoite piroplasm surface antigen (MPSA) (Katzer *et al.*, 2002). Tams-1, polypeptide characterized by variable molecular mass it is between 30 to 32 kDa (Katzer *et al.*, 1998). This gene encoded for protein of 281 amino acids (aa) containing a putative hydrophobic N-terminal signal peptide, which is highly variable. Several studies reported using of (Tams-1) target based PCR for sensitive and specific amplification of *T. annulata* DNA from blood of cattle and ticks D'Oliveira *et al.* (1995), Gubbels *et al.* (2000), Shiels *et al.* (1994, 2000) and Kirvar *et al.* (2000). The present study

aimed to compare between blood films and (Tams-1) target based PCR as a diagnostic tools for diagnosis of bovine theileriosis. Genetic polymorphism of Tams-1 gene in different isolates of *T. annulata* from different localities in Upper Egypt was monitored.

MATERIALS and METHODS

Since August 2010 till August 2013, a total number of 210 cattle belong to different localities in Upper Egypt were randomly selected to this study. Out of them, 11 positive samples were subjected to cloning, sequencing, amino acids translation and multiple sequences alignment. Two types of samples were collected from each animal; one blood sample was collected directly from the ear vein and used for preparation of blood films, another whole blood sample was collected on Ethylene Diamine Tetra-

Acetic acid (EDTA) as anticoagulant (1mg/1ml) by direct puncture of the jugular vein and marked with numbered labels then stored at (-20°C) till use in DNA-extraction Charles, (2002).

Blood film:

Thin blood films were prepared immediately post ear vein puncture then fixed and stained by Giemsa stain 8%. Three thin blood films from each animal were examined for detection of piroplasms or trophozoite (Coles, 1986).

Polymerase chain reaction (PCR):

(Tams-1) target based polymerase chain reaction was used for molecular diagnosis of *T. annulata* infection in examined blood samples.

DNA Extraction and polymerase chain reaction:

DNA extraction from whole blood was carried out according to manufacturer's instructions of commercial kits (according to QIA amp blood kit, Qiagen, Ltd, UK)

DNA amplification by polymerase chain reaction (PCR) using Tams-1 target-based primer:

For the standard PCR, primer **Tams1 F** (5' ATG CTG CAA ATG AGG AT) and **Tspms1 R** (5' GGA CTG ATG AGA AGA CGA TGA G), Amplifying a (785 bp) fragment of *T. annulata* 30 KDa major merozoite surface antigen gene Kirvar *et al.* (2000), Murat *et al.* (2008) and AL-Hosary, (2009).

Cycling conditions: 94°C for 5 min., followed by 37 cycles consisting of 1 min at 94°C, 1 min. at 55°C, 2 min. at 72°C and final extension step at 72°C for 10 min. longer then, the samples were stored at 4°C until use. The cycling condition carried out in thermocycler TECHNE TC-312. In addition to the samples positive control sample contain "DNA from *T. annulata* infected lymph" and negative control sample (DNase free water), then all were included in the amplification step.

Gel Electrophoresis: The chamber was connected to 75 volt power supply for 90 min., 10µl of each PCR product were separated by electrophoresis on 1.8% agarose gel (GX 040.90, Gen AGarose, L.E., Standard DNA /RNA agarose, Molecular Biology Grade, Inno-Train Diagnostic, D-61476, Kronberg/Taunus) Containing Ethidium bromide as 1 µl /ml electrophoresis buffer. The result obtained through high performance ultraviolet transilluminator,

the specific band were detected by using DOC-It® LS, Image acquisition-software, (UVP, INC, UK) at 785 bp.

Cloning of PCR product, DNA sequencing and data analysis

Cloning:

Ligation of the PCR product was carried out using pDrive Cloning vector according to manufacturer's instructions of QIAGEN Kit (Cat. NO.231224), Qiagen, Ltd, UK. Transformation by using QIAGEN Kit was carried out according to the manufacturer's instructions of Qiagen, Ltd, UK then Plating/Culture and the selection of clones was depended on (Blue / White Colony Selection).

Preparation of the samples for sequencing: The samples measured for detection the amount of dDNA by using eppendorf-Biophotometer, NO.6131 then adjust the sample concentration to 1µg/µl in total volume 15 µl in two tubes one for forward primer and the second for reverse primer.

Sequencing and data analysis: The amplicons were sequenced in both directions in the research center, Borstel, Germany by using Sanger 3730xl, Applied Biosystems (Life Technologies) as previously described by Holman, *et al.* (2005) and Schoelkopf *et al.* (2005). Tams-1 sequences were determined from alignments of 3 to 12 clones from each isolate and then the sequence results were subjected to Basic Local Alignment Search Tool (BLAST) similarity searches according to Altschul *et al.* (1990).

Sequences alignment and translation:

All the sequences were alignment together by using Clustal W, also translated into its amino acids (aa) by using Expasy.translate software and then alignment the obtained amino acids (aa) together by the same alignment program.

RESULTS

In the current study 210 cattle were randomly selected and subjected to examination for diagnosis of *T. annulata* infection.

Blood film and PCR:

The results revealed that the infection rate was 28.57% while the infection rate according Tams-1 target based PCR was 46.19% (Table 1 and Fig. 1, 2).

Table 1: Infection rate by using blood film and Tams-1 target based PCR

Giemsa stained thin blood film			Tams-1 target based PCR		
No. of tested animals	Positive	%	No. of tested animals	Positive	%
210	60	28.57	210	97	46.19

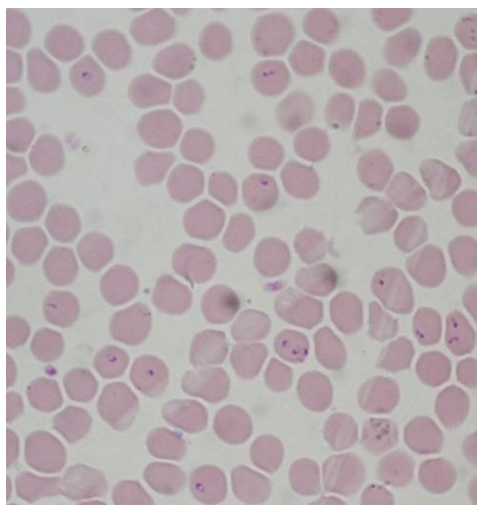


Figure 1: Blood film stained with Giemsa stain demonstrating intracellular trophozoit "signet ring" of *T. annulata* (X100)

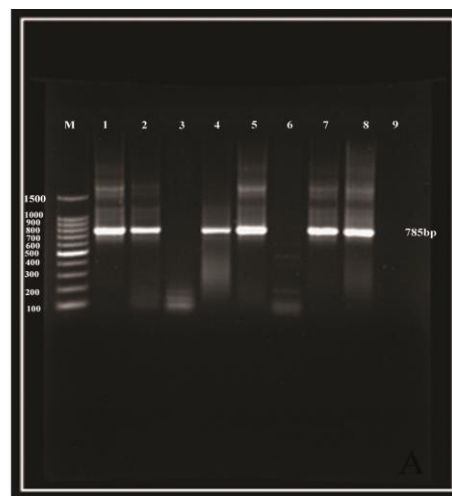


Figure 2: Agarose gel electrophoresis of (Tams-1) target based PCR amplified DNA from *T. annulata* infected and non-infected animals. M, 100 bp plus Marker DNA Lanes 1, 2, 4, 5 and 7 positive samples yielded 785 bp PCR product, lanes 3 and 6 Negative samples. Lanes 8 and 9 were the positive and negative control, respectively.

Sequences analysis:

The obtained results revealed that the local isolates of *T. annulata* are characterized by the polymorphism of Tams-1 gene. This finding obtained through alignment of the different obtained sequences together and with those in the gene bank by using (Clustal W) (Fig. 3). This was also confirmed after translation of the each sequence to its amino acids

(aa) by using Expsy.translate software and then alignment together (Fig. 4). The obtained sequences and their translated amino acids were deposited in the GenBank™ databases and available under accession numbers as following (GenBank™: KJ021626, GenBank™: KJ021627, GenBank™: KJ021628, GenBank™: KJ021629).

CLUSTAL 2.1 multiple sequence alignment

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KJ021627      ATGCTGCAAATGAGGATGAAAAGAAAAGGAAGGAAA-----AAAAGATGTTG
KJ021629      ATGCTGCAAATGAGGATGATAAGAAAAAGGAAGAAA AAAAGATGAAA AAAAGATGTTG
KJ021628      ATGCTGCAAATGAGGATGATAAGAAAAAGGAAGAAA AAAAGATGAAA AAAAGATGTTG
KJ021626      ATGCTGCAAATGAGGATGAAAAGAAAAGGAAGGAAA-----AAAGGATGTTG
*****

KJ021627      TTCTTGATGTTACTCTCACTTCATGTGATAAATGTAACCTTTAAAGTCTGTCGACTCTAACA
KJ021629      TTCTTGATGTTCTTGGCACTTCATGTGAGAATGTAGTCTTTCATACCTCCGACTCTAACA
KJ021628      TTCTTGATGTTCTTGGCACTTCATGTGAGAATGTAGTCTTTCATACCTCCGACTCTAACA
KJ021626      TTCTTGATGTTACTGCCACTTCATGTGAGAATGTAGCTTTGATAACCGCGACCTAACTT
*****

KJ021627      CCACTGAGTTAACTGTCTCGGATGGCTACCGTTTCAAGACCCTTAAGGTCGGAGACAAGA
KJ021629      CTACTGAATTACTTGTCAAAGACGGCTTCCGTTTCAAGACCCTTAAGGTCGGAGACAAGA
KJ021628      CTACTGAATTACTTGTCAAAGACGGCTTCCGTTTCAAGACCCTTAAGGTCGGAGACAAGA
KJ021626      CCGTCTGTGTTAACTGTCTCGGATGGCTACCGTTTCAAGACCCTTAAGGTCGGAGACAAGA
*

KJ021627      CCTTGTTCAATGTTGACACCTCAAAACATACCCAGTACAGGCATTCAAACCTAAGCATG
KJ021629      CATTTGTTCAATGTTGACACCTCAAAACATACCCAGTACAGGCATTCAAACCTAAGCATG
KJ021628      CATTTGTTCAATGTTGACACCTCAAAACATACCCAGTAAAGGCATTCAAACCTAAGCATG
KJ021626      CCTTATTCAATGTTGACACAGGAAAACATACCCAGTAAAGGCATTCAAACCTAAGCATG
*

KJ021627      AATCCGATGAGTGGTTCAGACTTAATCTTCACTGCTGCCAGCCAAAGATGTCAAGAAGA
KJ021629      AATCCGATGAGTGGTTCAGACTTAATCTTCACTGCTGCCAGCCAAAGATGTCAAGAAGA
KJ021628      AATCCGATGAGTGGTTCAGACTTATCTTCACTGCTGCCAGCCAAAGATGTCAAGAAGA
KJ021626      AATCCGAGGAGTGGTTCAGGCTTATCTTCACTGCTGCCAGCCAAAGATGTCAAGAAGA
*****

KJ021627      AGGGAGACAAGGAATATCTGAGGTCAAATTCGAGACCTACTACGATGAAGTCTTGTTC
KJ021629      AGGGAGACAAGGAATATCTGAGGTCAAATTCGAGACCTACTACGATGAAGTCTTGTTC
KJ021628      AGGGAGACAAGGAATATCTGAGGTCAAATTCGAGACCTACTACGATGAAGTCTTGTTC
KJ021626      AGGGAGACAAGGAATATCTGAGTCAAATTCGAGACCTACTACGATGAAGTCTTGTTC
*****

KJ021627      AGGGAAAATCAGCCAAGGAAGTAGATGCTTCCAAGTTCGAAGATACACTCTTGTTCACCT
KJ021629      AGGGAAAATCAGCCAAGGAAGTAGATGCTTCCAAGTTCGAAGATACAGCTTGTTCACCT
KJ021628      AGGGAAAATCCGCCAAGGAAGTAGATGCTTCCAAGTTCGAAGATACAGCTTGTTCACCT
KJ021626      AGGGAAAATCCGCCAAGGAAGTAGATGCTTCCAAGTTCGAAGATACAGCTTGTTCACCT
*****

KJ021627      CCTCCGCCTTCGGCAC TGAAAGATGTACACCTTAAAAAGGAATTTAAACCTTCCAAAG
KJ021629      CCTCCGCCTTCGGCAC TGAAAGATGTACACCTTAAAAAGGAATTTAAACCTTCCAAAG
KJ021628      CCTCCGCCTTCGGCAC TGAAAGATGTACACCTTAAAAAGGAATTTAAACCTTCCAAAG
KJ021626      CCTCCGCCTTCGGCAC TGAAAAAAGTACACCTTAAAAAGGAATTTAAACCTTCCAAAG
*****

KJ021627      TCACCTTCGACAAGAAAGAAGTCGGAAAAGCCAAACAATGCCAAGTATCTTGAAGTGTG
KJ021629      TCACCTTCGACAAGAAAGAAGTCGGAAAAGCCAAACAATGCCAAGTATCTTGAAGTGTG
KJ021628      TCACCTTCGAAAAGAAAGAAGTCGGAAAAGCCAAACAATGCCAAGTATCTTGAAGTGTG
KJ021626      TCTCTTCGAAAAGAAAGAAGTCGGAAAAGCCAAACAATGCCAAGTATCTTGAAGTGTG
*

KJ021627      TCTTTGTTGGTTCTGATTCGAAGAAGTTAGTCAAACCTTACTACTTCTATACCGGTGACT
KJ021629      TTTTGTGGTTCTGATTCGAAGAAGTTAGTCAAACCTTACTACTTCTATACCGGTGACT
KJ021628      TCTTTGTCAGTCTGATTCGAAGAAGTTCGCTCAGACTCAGACTACTTCTATACCGGTGACT
KJ021626      TCTTTGTCAGTCTGATTCGAAGAAGTTCGCTCAGACTCAGACTACTTCTATACCGGTGACT
*

KJ021627      CAAGGTTGAAGGAGACCTACTTCGAGCTTAAGGACGATAAGTGGGTCAAATGTCACAGG
KJ021629      CAAGGTTGAAGGAGACCTACTTCGAGCTTAAGGACGATAAGTGGGTCAAATGTCACAGG
KJ021628      CAAGGTTGAAGGAGACCTACTTCGAGCTTAAGGACGATAAGTGGGTCAAATGTCACAGG
KJ021626      CAAGGTTGAAGGAGACCTACTTCGAGCTTAAGGACGATAAGTGGGTCAAATGTCACAGG
*****

KJ021627      CAGATGCAAAACAAGGCTTGAATGCCATGAACCTCCTCGGTCACCGATTACAACCAG
KJ021629      CAGATGCAAAACAAGGCTTGAATGCCATGAACCTCCTCGGTCACCGATTACAACCAG
KJ021628      CAGATGCAAAACAAGGCTTGAAGCCATGACCTCCTCGGTCACCGATTACAACCAG
KJ021626      CAGATGCAAAACAAGGCTTGAATGCCATGACCCCACTCGGTCACCGATTACAACCAG
*****

KJ021627      TTGTCGACAAGTTCTCCCTTGCAGTCTTCGCCTCAGTACTCATCGTCTTCTCATCAG
KJ021629      TTGTCGACAAGTTCTCCCTTGCAGTCTTCGCCTCAGTACTCATCGTCTTCTCATCAG
KJ021628      TTGTCGACAAGTTCTCCCTTGCAGTCTTCGCCTCAGTACTCATCGTCTTCTCATCAG
KJ021626      TTGTCGACAAGTTCTCCCTTGCAGTCTTCGCCTCAGTACTCATCGTCTTCTCATCAG
*****

KJ021627      TCCAATCAC
KJ021629      TCCAATCAC
KJ021628      TCCAATCAC
KJ021626      TCCAATCAC

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Figure 3: Sequences' alignment for *T. annulata* Tams-1 gene.

CLUSTAL 2.1 multiple sequence alignment

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KJ021626      MFKKKGDKKEYSESKFETYDVLFLFKGSAKELDVSKFEDTALFTASAFGTGKKYTFKKDF
KJ021628      MFKKKGDKKEYSESKFETYDEVLFKFKGSAKELDVSKFEDTALFTASAFGTGKKYTFKKDF
KJ021627      MFKKKGDKKEYSEVKFETYDEVLFKFKGSAKELDASKFEDTSLFTSSAFGTGKMYTFKKEF
KJ021629      MFKKKGDKKEYSEVKFETYDVLFLFKGSAKELDVSKFEDTALFTSSAFGTGKMYTFKKEF
*****
KJ021626      KPSKVLEKKEVGVKPNNAKYLDFVVFVSDSKKVVRLDYFYTGDSRLKETYFELKDDKVV
KJ021628      KPSKVTFEKKEVGVKPNNAKYLDFVVFVSDSKKVVRLDYFYTGDSRLKETYFELKDDKVV
KJ021627      KPSKVTFDKKEVGVKPNNAKYLEVVVVFVGSDDSKLVKLYYFYTGDSRLKETYFELKDDKVV
KJ021629      KPSKVTFDKKEVGVKPNNAKYLEVVVVFVGSDDSKLEVKLYYFYTGDSRLKETYFELKDDKVV
*****
KJ021626      QMSQADANKALNAMDPWSSDYKPVVDKFSPLAVFASVLIVFSSVQS
KJ021628      QMSQADANKALNAMSSWLA DYKPVVDKFSPLAVFASVLIVFSSVQS
KJ021627      QMSQADANKALNAMSSWSTDYKPVVDKFSPLAVFASVLIVFSSVQS
KJ021629      QMTQADANKALNAMSSWSTDYKPVVDKFSPLAVFASVLIVFSSVQS
**:*

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Figure 4: The alignment of translated (aa) of *T. annulata* Tams-1 gene

DISCUSSION

The infection rate with *T. annulata* of the examined cattle was 28.57% by thin blood film and 46.19% by Tams-1 target based PCR. These obtained results revealed that molecular technique was more sensitive it was in agreement with D'Oliveira *et al.* (1995), Aktas *et al.* (2002, 2006), Tabidi *et al.* (2006), Azizi *et al.* (2008), Durrani and Kamal, (2008) and AL-Hosary, (2009). They reported that blood smear is a quick and cheap for diagnosis of *T. annulata* infection in cattle, and PCR test was more sensitive and specific test.

Regarding to the genetic polymorphism of (Tams-1) gene that responsible for *T. annulata* Merozoite piroplasm surface antigen, the results revealed that Tams-1 gene from different *T. annulata* isolates are differ from each other. This agree with Manuja *et al.* (2006) who reported that (Tams-1) is a polymorphic gene and it was studied amongst four *T. annulata* isolates collected from three different parts of India. The four isolates of *T. annulata* were different from each other and may be express different antigenic determinants on their cell surface. Furthermore, Altay *et al.* (2007) and Esmaelizad *et al.* (2001) reported that the polypeptide (Tams1) is a highly variable immunodominant major merozoite piroplasm surface antigen of the protozoan parasite *T. annulata* and its associated with the evasion of the bovine immune system. Another possible explanation for the high levels of genetic diversity in Tams-1 gene among *T. annulata* isolates was reported by Gubbels *et al.* (2000) who concluded that it may be due to random mutation of nucleotides during asexual reproduction. This makes it difficult to use in vaccination or serological diagnostic assays. It concluded that Tams-1 gene merozoite surface antigens in *T. annulata* characterized by high level of genetic polymorphism making it unfit for serological diagnostic tools or vaccine production.

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دراسات تشخيصيه وجينيه للثيليريا الحلقية مع اشارته خاصه للتنوع الجيني في انتجين التامز-1 للثيليريا الحلقية

أميره عادل طه الحصري ، ليلى صلاح الدين أحمد ، اورليكا سيتر

Email: amiraelhosary@ yahoo.com

استهدفت الدراسة الي استخدام الطريقة التقليدية متمثلة في المسحات الدموية المصبوغة بصبغة الجيمسا بالإضافة الي تفاعل البلمرة المتسلسل باستخدام بادئ التامز-1 لتشخيص الاصابه بمرض ثيليريا الماشيه في الابقار في صعيد مصر. ووضحت النتائج ان طريقة المسحة الدموية مازالت تتمتع باهميه اكلينيكيه ولكن استخدام طريقة تفاعل البلمرة المتسلسل باستخدام بادئ التامز-1 هي الاكثر حساسيه. بادئ التامز-1 مستخلص من جين التامز-1 وهو جين مسئول عن الانتجين السطحي الخاص بالطفيل. وكان من أحد اهداف هذه الدراسة ايضا تتبع هذه الخاصيه في جين التامز-1 المعزول من طفيل الثيليريا الحلقية المعزوله من الابقار في صعيد مصر وتم تأكيد هذا التنوع الجيني وتم نشر المتواليات الجينيه وتتابعات الاحماض الامينييه (البروتين) الخاص بها في بنك الجينات تحت ارقام الانضمام التاليه: KJ021266, KJ021267, KJ021268, KJ021269 ان هذا الجين له القدره علي التغير من نفسه بالشكل الذي يجعل البروتين الناتج عنه مختلف وبالتالي يعمل علي حمايه الطفيل من الاجسام المضاده التي ينتجها الجهاز المناعي الخاص بالحيوان المصاب ولذلك لا ينصح باستخدام هذا الجين في طرق التشخيص السيرولوجيه او التحصين ضد المرض.