التحليل الجزيئي للجين (A2) بعد علاج الجرذان المصابة تجريبيا بالليشمانيا الحشوية

Molecular analysis of A2 gene after treated experimental infected rats with visceral leishmaniasis

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Summary

The present study has been carried out at College of Veterinary Medicine, Al-Qadisiya University, Iraq for diagnosed the visceral leishmaniasis by qPCR reaction and to investigate the effect of treatment with Pentostam ™ (100 mg /ml) on mRNA expression level of A2 gene in blood and bone marrow samples of female rats using Reverse Transcriptase –Polymerase Chain Reaction . Blood samples were collected from children infected by visceral leishmaniasis, then injected in the peritoneal cavity of Fourty *Wistar* female rats (weighted 250 ± 2 g.) were assigned into two groups; control group(10rats) and treated group (30 rats) . After 8-10 days blood samples collected directly from the heart for diagnosis the infection using quantitative Polymerase Chain Reaction , also collected blood and bone marrow samples from control group to detect A2gene expression by Reverse Transcriptase – Polymerase Chain Reaction. The second group was treated with pentostam at dose 25 mg / kg for 30 days and divided into four weeks, and in each week collected blood and bone marrow samples to check the effect of treatment on mRNA expression level of A2 gene. In all of the experimental blood samples, all the results were positive when diagnosed by quantitative Polymerase Chain Reaction . Reverse Transcriptase –Polymerase Chain Reaction results which relied on the relative quantification of A2 gene expression normalized by gene expression levels of housekeeping gene (Glyceraldhyde 3-phosphate dehydrogenase(GAPDH)),The relative quantification results detected the fold changes in A2 gene levels of blood and bone marrow samples of rats treated with pentostam. The level of A2gene expression of blood samples was in the first week 0.24 fold, in the second week 0.08 fold , in the third week 0.02 fold and in the fourth week 0.006 fold ,all of that was lower than control sample. While in bone marrow samples level of A2 gene expression was in the second week 0.09 fold , in the third week 0.04 fold and in fourth week 0.03 fold, all of that lower than control sample.

It can be concluded that the pentostam has active effect on virulence factor (A2gene) ,also the parasite remains dormant in bone marrow samples even after treatment and possibly return to virulence in the case of any immune inhibition .

Introduction

Leishmaniases are protozoal diseases caused by a hemoflagellate of the genus *Leishmania* and transmitted by sand flies (1). The pathologies of leishmaniasis range from fatal infection of the visceral organs to mild cutaneous infection (2). Natural transmission of *leishmania* is carried out by a certain species of sandfly of the genus *Phlebotomus* (Old World) or *Lutzomyia* (New World) and canids serve as reservoir hosts(3) and can be transmitted congenitally or parentally (4). The A2 gene family was first identified in *L. donovani* because the A2 transcripts are abundant in the amastigotes, but hardly detectable in the promastigotes, and is located mainly in the cytoplasm (5,6).The A2 protein from *Leishmania donovani* is expressed at high levels in axenic amastigote cultures and at low levels in promastigote cultures (7). The A2 gene is essential for survival of this parasitic protozoan in its mammalian host (7).The A2genes, however, are not present in all species of *Leishmania* and are absent in *L.major* (8).The diagnosis of leishmaniasis, is performed by direct visualization of amastigotes using microscopic examination of stained material, by isolation of the parasite in culture, and by detection parasite using serological methods. Microscopic examination of stained-Giemsa slides, though rapid and low-cost, has limited sensitivity, particularly in chronic lesions (9).In vitro culture techniques, while more sensitive, are susceptible to microbiological contamination, and are hampered by the particular growth requirements of different strains (10). Recently PCR based methodsand DNA probes are highly sensitive, compared with standard methods and are considered valuable for diagnosis (11). PCR, particularly quantitative real-time PCR, can be more sensitive than serology, and has high specificity (12).The primary treatment against leishmaniasis includes pentavalent antimonials, also other drugs such as amphotericin B, pentamidine, miltefosine can be used (13).The drugs used in leishmaniasis treatment present several problems, including high toxicity and many adverse effects, leading to patients withdrawing from treatment and emergence of resistant strains (14).The treatment of leishmaniasis probably seldom eradicates all parasites in tissue macrophages; nevertheless, most T cell-intact

patients show long-lasting clinical cure after treatment, despite residual intracellular infection, which can provoke the post-treatment relapse of infection (15).

Materials and Methods

Experimental animals

Old female Wister rats (average weight was 250 ± 2g), born at the animal house of the College of Veterinary Medicine, AL-Qadissiya University. The rats were reared under controlled ambient temperature (20-25C°) and fed on standard laboratory diet (19% protein ratio and 3000 kilocalories energy) and drinking water *ad lebitum*.

Laboratory animals inoculation and collection the samples

After collected 40 blood samples from children injected in abdominal cavity of rats, injection is usually done without anesthetized the rats by raising the animal by the left hand from the back and the head for the top, then injected into the peritoneal cavity by sterile syringe (1 ml) (16) . After 8-10 days collected blood directly from heart after anesthetized the rats using 0.2 Ketamine and 0.1 xylazine for diagnosed the infection by quantitative Polymerase Chain Reaction and collected blood and bone marrow samples from control group by cutting the bone from both sides ,after that begin the process of washing the bone from inside by inserting syringe needle which contain distilled water injected into the bone and put sterile tube at the bottom of the bone to get on the fluid bone marrow of the femur to detect A2 gene expression by Reverse Trancriptase - Polymerase Chain Reaction, after that, 30 rats treated with pentostam at dose 25mg/kg for 30 day I/m (17) . Then collected blood and bone marrow samples every weeks for detect the effect of treatment on A2gene expression level by RT-PCR .

Primers

Two primers were used in this study, first primer used for GAPDH gene as Housekeeping gene and second primer used for A2gene as target gene. These primers were designed by using NCBI- Gene Bank data base and Primer 3 design online, the primers used in quantification of gene expression using qRT-PCR techniques based SYBER Green DNA binding dye, and supported from (Bioneer, Korea) company.

Table (1): The Primers and their sequences, gene bank accession number, and references.

Primer	sequences		Accession number
GAPDH	F	CTCACAGTGCGTCTGGAGAA	AB608734.1
	R	TCGGTGTAGCCGAGAATACC	
A2	F	CGCTGATGTGCTGACTTGTT	S69693.1
	R	CGGGGGCACTGAGAATAATA	

Molecular study

Diagnostic Real-Time PCR (qPCR)

qPCR technique was used for amplification of conserved region in GAPDH gene that used for detection of *Leishmania donovani* in blood samples of rats. This technique was done according to method described by(18).

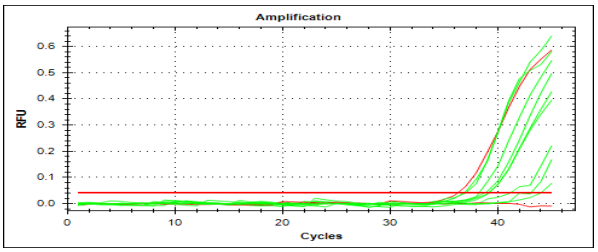
Quantitative Reverse Transcription Real-Time PCR

qRT-PCR technique was used for quantification of A2 gene expression levels in infected control and treatments groups in blood and bone marrow of rats treated with Pentostam ™ (Sodium stiboglucanat). As well as Housekeeping gene(GAPDH) was used for normalization of A2 gene expression. This technique was done according to method described by (19).

Results

Quantitative Real Time PCR diagnostic result

To give the absolutely diagnosis for Visceral leishmaniasis in the blood sample from experimental infected rats used Quantitative Real -Time PCR using housekeeping gene (GAPDH). Fig(1,2,3) shown amplification and melt curve and melt peak of *L. donovani* respectively.there was different positive reaction cycle extend from 35-40 cycle.



Figure(1):The Real -Time PCR Amplification plot of *L. donovani* genomic DNA template concentrations .

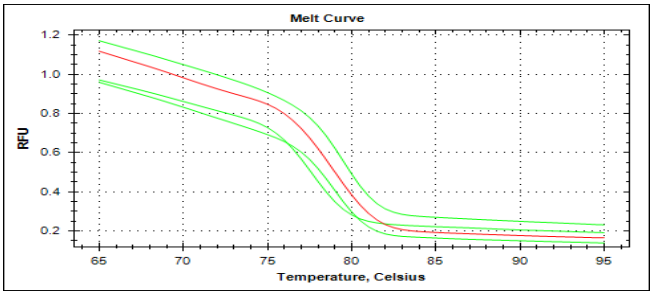


Figure (2) : The Real -Time PCR melt curve of *L. donovani* genomic DNA template concentration

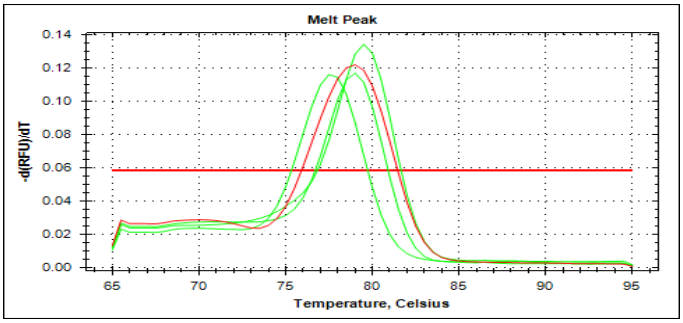


Figure (3) : The Real -Time PCR Melt peak of *L. donovani* genomic DNA template concentrations .

Quantitative reverse transcription Real-time PCR result

RT-qPCR was used for determination of A2gene expression in treated rats for blood and bone marrow samples in different week period of infection , as well as housekeeping gene (GAPDH) was used for normalization of A2 gene expression in blood and bone marrow samples. Fig(4,5,6,7,8,9,10).

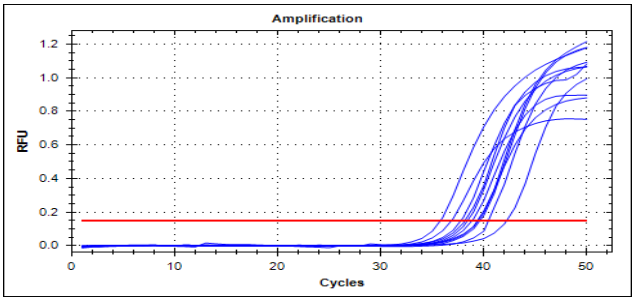


Figure (4): The Real- Time PCR Amplification plot of A2 gene in blood samples.

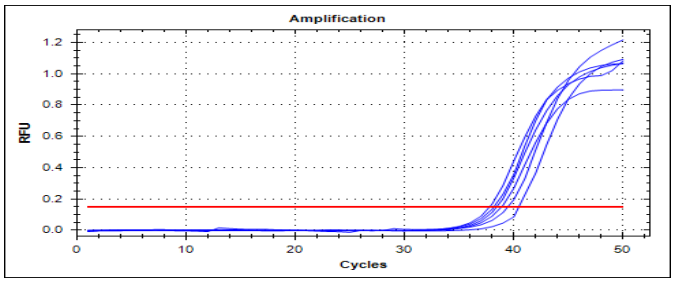
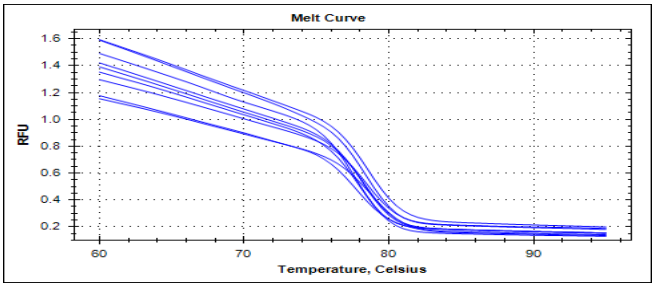


Figure (5): The Real- Time PCR Amplification plot of A2 gene in bone marrow samples.



Figure(6): The Real -Time PCR melt curve of A2 gene .

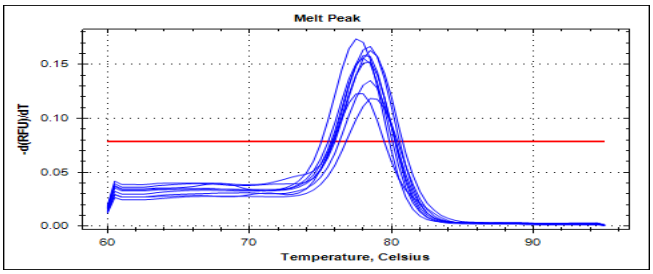
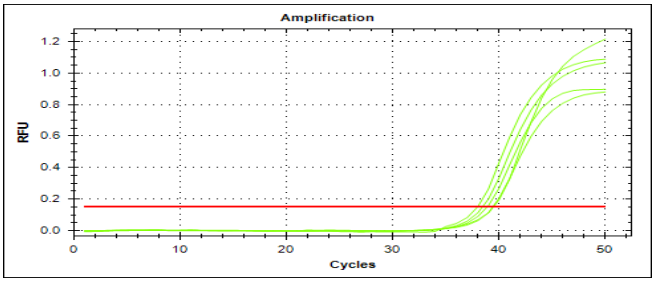
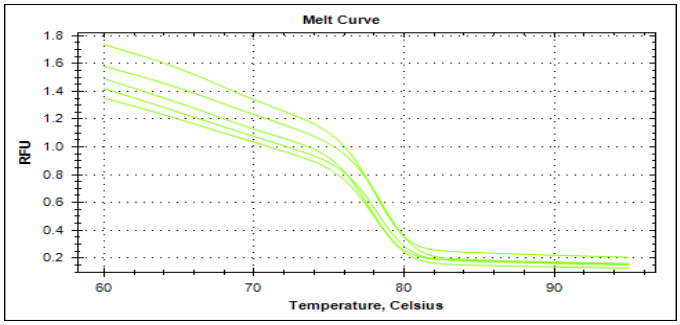


Figure (7): The Real -Time PCR Melt peak of A2 gene



Figure(8): The Real- Time PCR Amplification plot of housekeeping gene (GAPDH).



Figure(9): The Real -Time PCR melt curve of housekeeping gene .

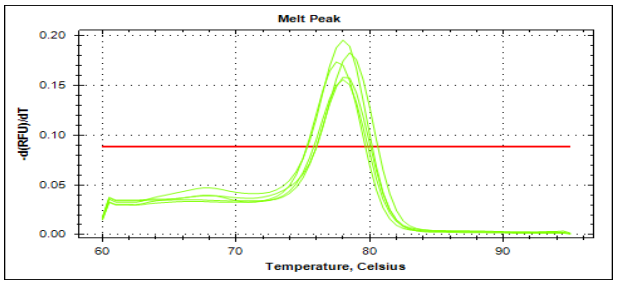


Figure (10): The Real -Time PCR Melt peak of housekeeping gene.

Relative quantification of target gene expression:

To calculate the relative expression of target gene (A2 gene) in blood and bone marrow samples, the $2^{-\Delta\Delta CT}$ (livak) method used by normalize gene expression of target gene(A2) with expression of housekeeping gene (GAPDH) as reference gene. At first, the threshold cycle number of target gene normalized to that of reference gene in all treatment groups and calibrator (control groups). Second, the ΔCt of treatment groups and the ΔCt of control group is normalized, and finally the expression ratio (fold change) was calculated ($2^{-\Delta\Delta CT}$).

Relative quantification of A2 gene expression in blood samples

Figure (11) show the mean of A2gene expression after treatment in the first week is up- regulation (0.24) when compare with the fourth week (0.006).

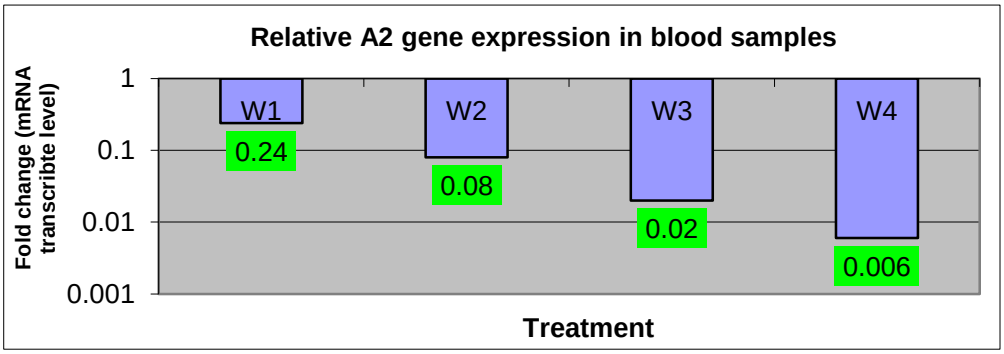
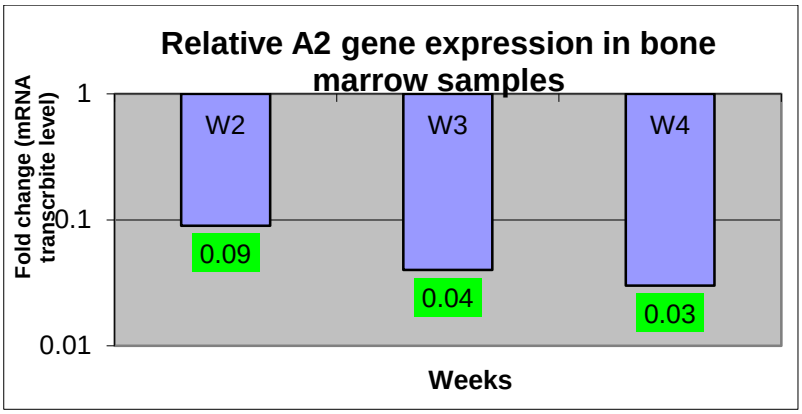


Figure (11): Histogram explains relative A2 gene expression in blood samples .

Relative quantification of A2 gene expressionin bone marrow samples:

Figure (12) show the mean of A2 gene expression after treatment in the second week is up- regulation (0.09) when compare with the fourth week (0.03).



Figure(12):Histogram explains relative A2 gene expression in bone marrow samples

Disscussion

diagnosis of visceral leishmaniasis

Because the signs and symptoms of other pathologies can mimic those of VL and given that early diagnosis is decisive in reducing the morbidity and mortality rates , the use of molecular methods for diagnosis is warranted . Among the methods available , real time PCR is particularly advantageous , given its effectiveness for samples containing a small number of parasites and in cases in which differential diagnosis can be crucial in view of the wide range of clinical manifestation (20) .

In this study it was describe the establishment of a real-time PCR assay for detection of visceral Leishmaniasis in clinical samples. The results oo present study show positive result from all experimental infected rats samples which gave positive result after diagnosed by rapid rk39 test**.** Similar results were documented by (12 , 21, 22). (23) Using peripheral blood samples from 15 dogs that were positive for VL, qPCR sensitivity was 100% .

(24) also compared the conventional PCR with qPCR , using spleen and liver samples from *L. infantum*-infected mice, they found qPCR was more sensitive than PCR. Wortmann and his team investigated diagnosis of leishmaniasis using qPCR, They showed a sensitivity was 96% for this test while that of direct microscopy and culture was 30 and 33% respectively (25). (18) developed a quantitative assay that was able to discriminate different *Leishmania* species, but accuracy was weak when parasitemia was under 100 parasites/ml . As a consequence, such a test cannot be used for follow-up testing of treated patients, while the qPCR allows the possibility of quantification at 1 parasite/ml. Mary and her group found the qPCR is more sensitive than conventional PCR in diagnosis of *Leishmania* species, they

found Real-Time PCR was sufficiently sensitive to detect as little as 0.001 parasite in blood samples (20).

This study found the qPCR assay have 100% sensitivity from all experimental infected rats because this test allows possible to discriminate the parasite in blood.

Quantitative Real-Time Reverse Transcription PCR Assay

This technique has major advantages of extremely rapid with results often obtained within 1 hour after DNA processing, it is less labor intensive (there are no gels to run and more samples can be processed at one time), there is less risk of contamination (the PCR tubes remain closed during the entire process), and it is highly specific by using a combination of amplification primers to detect a target gene compared to the conventional quantification methods as conventional PCR(20,22),therefore, to obtain a reliable quantification method , each real time–PCR method has to be optimized taking into account the different successive step in this multistep procedure such as lysis of leishmania cell , RNA extraction, DNase treatment, cDNA synthesis and finally real time PCR.

Relative gene expression

The relative quantification of A2 gene expression in blood and bone marrow of rats was determined using the $2^{-\Delta\Delta CT}$ (Livak method) . This method developed by (26) who referred that this method assume both target and reference gene are amplified with Real – Time PCR efficiencies near to 100% within 5% of each other . Therefore , the gene expression was calculated by this method .Fold changes were represented relative gene expression of target gene (A2) that normalized to reference gene (GAPDH) , the normalization used reference gene is very important to estimate the fold change in gene expression levels , according to (27) who indicated that correct measurement of gene expression levels in quantitative real-time reverse transcription PCR (RT-PCR) is accomplished used normalization strategy that involves standardization to a single constitutively expressed control gene.

To our knowledge this is the first study describing the isolation and characterization of A2 gene (virulence factor) after treated the infected rats with Pentostam ™. all our results demonstrate good effect of treatment of all experimental infected rats. Our results demonstrated Relative A2 gene expression in blood sample after treatment is 0.24 fold in first week, 0.08 fold in second week , 0.02fold in third week and 0.006 fold in fourth week,

all of that lower than control sample . While in bone marrow samples was 0.09 fold in second week, 0.04 fold in third week and 0.03 fold in fourth week, all of that lower than control sample .

The Pentostam is the drug which may effect on DNA of parasite and gave the recovery result clinically but the presence of A2 gene expression in Quantitative Real-Time Reverse Transcription PCR Assay explain the high sensitivity of this test to detect the genes of parasite and detection the relapse of VL in patients after 6–12 months after treatment through immune deficiency (28).

The result showed in the mean of A2 gene between organs, the lower mean of it found in the blood 0.006 fold change (mRNA transcript level) than the mean of A2 in the bone marrow samples(0.03 fold) , which may be due to found the parasites dormant in the reticulo-endothelial cells of this organ or may be return of virulence after lower the immunity of the patients and occurrence the relapse of VL.

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الخلاصـــــــــة

أجريت الدراسة الحالية في كلية الطب البيطري بجامعة القادسية لتشخيص الليشمانيا الحشوية بواسطة تقنية تفاعل سلسلة البلمرة الكمي وكذلك للتحري عن تأثير البنتوستام 100 ملغم / مل على التعبير الجيني لجين (A2) في الدم ونخاع العظم في إناث الجرذان باستخدام تقنية تفاعل سلسلة البلمرة في الوقت الحقيقي الكمي. تم جمع عينات الدم من الأطفال المشكوك بإصابتهم بالليشمانيا الحشويه بعد ذلك حقنت في التجويف ألبطني للجرذان(٤٠ جرذاً) حيث قسمت الى مجموعتين السيطرة (١٠) ومجموعة معالجة (٤٠)، بعد ٨-١٠ أيام تم سحب الدم مباشرة من القلب لتشخيص الاصابة بواسطة تفاعل سلسلة البلمرة الكمي ، كذلك جمعت عينات الدم ونخاع العظم من مجموعة السيطرة لقياس مستوى التعبير الجيني لجين (A2). المجموعة الثانية تم معالجتها باستخدام البنتوستام وكانت الجرعة 25ملغم/كيلو في العضلة لمدة 30 يوم حيث قسمت إلى أربع أسابيع وفي كل أسبوع يتم جمع عينات الدم ونخاع العظم للتحقق من مدى تأثير العلاج على التعبير لجين (A2) في كل أسبوع باستخدام تقنية تفاعل سلسلة البلمرة في الوقت الحقيقي الكمي. أظهرت نتائج التشخيص باستخدام تقنية تفاعل سلسلة البلمرة الكمي نتيجة موجبة لجميع عينات الدم التجريبية. أما نتائج اختبار تفاعل سلسلة البلمرة في الوقت الحقيقي الكمي فقد اعتمدت على العد النسبي الذي يمثل التعبير الجيني لجين A2 بواسطة التعبير الجيني لجين المحافظ. أن نتائج العد النسبي الذي يتعلق بقياس مستويات التغير المضاعف في التعبير الجيني لجين A2 في عينات الدم ونخاع العظم للجرذان المعالجة ، قد أشارت بان مستوى التعبير الجيني لعينات الدم بعد العلاج في الأسبوع الأول0.24 أضعاف وفي الأسبوع الثاني0.08 أضعاف وفي الأسبوع الثالث 0.02 أضعاف وفي الأسبوع الرابع 0.006 أضعاف ، جميعها كانت أوطا من عينة السيطرة ،بينما مستوى التعبير الجيني لعينات نخاع العظم بعد العلاج في الأسبوع الثاني 0.09 أضعاف وفي الأسبوع الثالث 0.04 أضعاف والأسبوع الرابع 0.03 أضعاف، جميعها كانت أوطا من عينة السيطرة. يستنتج من نتائج الدراسة الحالية بان البنتوستام له تأثير فعال على عامل الضراوة (جين A2)،كما إن الطفيلي يبقى ساكنا في نخاع العظم حتى بعد العلاج ومن المحتمل أن يعود للضراوة في حالة حدوث أي تثبيط مناعي .