In vitro Activity of *Bacillus thuringiensis* (H14) 43 kDa Crystal Protein Against *Leishmania major*

[1]Hanan A. El-Sadawy, [2]Hussien A. Abou El-Hag,
[3]Janette M. Georgy, [4]Shaaban S. El Hossary and [5]Hala A. Kassem

[1]Department of Parasitology and Animal Diseases, National Research Center, Dokki, Egypt
[2]Department of Microbiology and Immunology, National Research Center, Dokki, Egypt
[3]Department of Zoology, College for Woman Arts, Science and Education, Ain Shams University, Egypt
[4]Research and Training Center on Vectors of Diseases, Ain Shams University, Egypt
[5]Department of Environmental Basic Sciences, Institute of Environmental Studies and Research,
Ain Shams University, Egypt

Abstract: Leishmaniases are caused by hemoflagellate protozoan which belongs to genus *Leishmania* order Kinetoplastida, family Trypanosomatidae, that infect vertebrate hosts through the bites of sand fly female *Phlebotomus* spp. Antimonials are still considered as the drug of choice for the treatment of leishmaniases. However, using microbial biological control agents as alternative strategy was developed. *Bacillus thuringiensis* (*B.t.*) crystal proteins are one of choice in this study. *Bacillus thuringiensis* is an endospore-forming, Gram-positive, soil bacterium that produces crystalline, proteinaceous inclusions, toxic mainly to lepidopteran, coleopteran and dipteran insects. The aim of this study is to investigate the effect of active non truncated core toxin of *B.t.* serovar *thuringiensis* (H14) 43 kDa for their activity against *Leishmania major* promastigotes. *Bt serovar. thuringiensis* separated crystalline proteins were dissolved, fractionated and the untruncated protein core was purified. The dissolved purified active protein fraction was examined electrophoretically by 7.5% native gel and 12% SDS-PAGE and its molecular weight was found to be 43 kDa. This active protein fraction was bioassayed against *L. major* promastigotes suspension (7×10^7 promastigotes ml^{-1}) in final concentrations ranging from 100 - 0.78 µg ml^{-1} aseptically in replicates. The bioassay of protein fraction against *L. major* promastigotes showed that its LC$_{50}$ is 4.95 µg ml^{-1}. The cytopathological examination of the treated promastigotes revealed that the pathological changes passed through degenerative changes that started with shortening, swelling and ended with complete death. Our results point to *Bt* (H14) 43 kDa crystal protein as a potential candidate for further investigation and potential application as a therapeutic agent against Leishmaniases.

Key words: *Bacillus thuringiensis* · *Leishmania major* · parasporal inclusions proteins · vector borne diseases

INTRODUCTION

The leishmaniases are caused by homoflagelate protozoan called *Leishmania* and are transmitted to animals and humans by the bite of phlebotomine sandflies. Leishmaniases are endemic in 88 countries on four continents and epidemics are frequent in the tropics and subtropics especially in the developing countries [1]. Leishmaniases present a broad clinical spectrum, ranging from asymptomatic and self-healing infections to those causing significant mortality [2]. Zoonotic cutaneous leishmaniasis (ZCL) due to *Leishmania major* produces skin lesions in the exposed areas of the human skin, in lieu of the bite of an infected sand fly. The zoonotic cycle of the disease involves: infected reservoir host-sand fly-human. Around one million cases of cutaneous leishmaniasis occur annually. In the Middle East, despite huge underreporting, more than 350 000 cases of cutaneous leishmaniasis occur annually and this trend is increasing in many countries. The disease has

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Corresponding Author: Dr. Hussein A. Abou El-Hag, Department of Microbiology and Immunology, National Research Center, Dokki, Egypt

been neglected as a major public health problem because it is not a dangerous killing disease. About 15% of patients have severe forms of cutaneous leishmaniasis requiring lengthy and frequently ineffective treatment [3]. Pentavalent antimonials are still the drug of choice for the treatment of leishmanises. These compounds are toxic, expensive and require long term treatment. The emergence of drug resistance parasites has been reported [2,4,5] which necessitates the search of new antileishmanial agents. Several new antileishmanial compounds have been investigated. These include diaryl derivatives [6]; quinoline compounds [7] and bupavaquone [8]. These compounds are synthetic chemicals that prove toxic effect to humans. Attempts have been also made to search for new sources of anti-leishmanial compounds from different protein groups in venoms of scorpions [9], *Jacaranda puberula* leaves [10], rattlesnakes [11] and eugenol-rich essential oil derivative [12].

Bacillus thuringiensis is a gram positive bacterium that produces crystal proteins during sporulation. It is toxic to lepidopterous, coleopeterous and dipterous insects in addition to mites, nematodes, protozoa and flukes [13-15]. *Bacillus thuringiensis* crystal proteins are nontoxic to vertebrates and many beneficial arthropods because it binds to the appropriate receptor on the surface of mid gut epithelial cells. Any organism that lacks the appropriate receptors in its gut cannot be affected by *B.t.* [16,17]. *Bacillus thuringiensis* crystal proteins showed *in vitro* cytotoxicity against human cancer cells and leukemic T cells [18]. More recently, *Xu et al.* [19] demonstrated that the *B.t.* crystal proteins can protect plasmodium-infected mice from malaria. In the light of recent discoveries of non-conventional targets of *B.t.* toxins, this study attempts to investigate the effect of an active none truncated core toxin of *Bacillus thuringiensis* serovar *thuringiensis* (H14) 43 kDa crystal protein for their activity against *Leishmania major* promastigotes to introduce it as a possible therapeutic antiparasitic agent. To the best of our knowledge, no antileishmanial *B.t.* strains have yet been reported.

MATERIALS AND METHODS

***Leishmania* parasites:** The parasites used in this study, was *Leishmania major* isolated from rodents collected from Nekhel during field trips carried out during summer of 1996 and have been maintained *in vitro* culture.

Bacterial Strain: *Bacillus thuringiensis* isolate, serovar *thuringiensis* (H14) used in this study was obtained from

National Microbial Bank (Faculty of Agriculture, Ain Shams University, Egypt). The isolate was grown at 28°C on nutrient agar (pH 7.6) consisting of meat extract (10 g), polypeptone (10 g), NaCl (2 g), agar (15 g) and distilled water (1000 ml).

Fermentation Process: Ten colonies of 24h age *Bt* were picked up and inoculated in trypticase soya broth (first passage) for 24h at 25°C. The fermentation process was carried out according to Mohd-Saleh *et al.* [20] with a modification in the run fermentation medium by adding a sporulation enhancer salts. The used run fermentation medium was Brain Heart Infusion broth containing salt additives (0.3, $MgSO_4.7H_2O$; 0.02g, $FeSO_4.7H_2O$; $0.02.ZnSO_4.7H_2O$; 1.0g, $CaCO_3$) per liter.

Separation of parasporal inclusion crystals: Parasporal inclusions were purified by biphasic separation technique using Na_2SO_4 and carbon tetrachloride according to Pendleton and Morrison [21]. Purified inclusions were washed three times with distilled water via centrifugation at 20,000 g at 4°C for 30 min. The purified parasporal inclusion crystal proteins were precipitated by acetone and were dried by desiccation overnight at room temperature and then stored at 4°C until use.

Solubilization and Extraction of *B.t.* serovar *thuringiensis* (H14) crystal proteins: Solubilization of 1g purified parasporal inclusion crystal proteins was performed in 1M NaOH (pH 12) at 25ºC for 24h with stirring [22]. The soluble protein solution was adjusted to pH 4 by 1N HCl. The precipitated protein fraction was separated by centrifugation at 25,000 g for 30 min at 4°C; then dissolved in 100 ml phosphate buffer saline (PBS) pH 7.2. The solubilized protein fraction was separated by centrifugation at 25,000 g for 30 min at 4°C. The total dissolved protein in the supernatant was precipitated in 80% saturated solution of ammonium sulfate anhydrous overnight with steering at 4°C. The precipitated protein fraction was dissolved in 80 ml distilled water and dialyzed overnight against 2X distilled water 2L each at 4°C. The resulted protein fraction was adjusted to total volume 100 ml. The active protein fraction between 30 and 50 kDa was separated using Vivaspine concentrator 20 by centrifugation at 2000 rpm at 4°C for 2h each. The protein recovery was monitored in each step of purification.

Protein determination and Gel Electrophoresis (PAGE): Protein concentration was measured by the method of Bradford [23] using bovine serum albumin as standard.

SDS-PAGE was performed as described by *Laemmli* [24], using 4% stacking and 12% resolving gels with Bio-Rad Minigel system at 50 V for 10 min and 100 V for 90 min. After electrophoresis, the gels were stained with 0.1% (W/V) coomassie blue R250. The molecular masses of the parasporal body proteins were estimated by comparison with those of the following proteins subjected to SDS-PAGE: lysozyme (14.4 kDa), soybean trypsin inhibitor (21.5 ktDa), carbonic anhydrase (31 kDa), ovalbumin (43 kDa), bovine serum albumin (66 kDa), phosphorylase b (92 kDa), P-galactosidase (116 kDa) and myosin (200 kDa). The number and purity of isolated protein fraction were estimated and confirmed by Native PAGE 7.5% as described by *Kishimoto et al.* [25].

Bioassay of *B.t.* (H14) against *Leishmania* promastigotes: About 10×10^7 promastigotes were used for each toxin dilution. Promastigotes suspension was centrifuged and resuspended in Schneider insect medium in final concentration of 7×10^7 promastigotes ml^{-1} [26]. Double fold dilution of toxic protein fraction was resuspended to promastigote suspension in final concentrations (100, 50, 25, 12.5, 6.25, 3.12, 1.56 and 0.78 μg ml^{-1}) in 96-well tissue culture plate under aseptic conditions. Each concentration was tested in triple trials under similar conditions. The inoculated plate was incubated at 25°C for 24h in humidified incubator. The incubated promastigotes were tested microscopically for cytopathological changes.

Antiparasitic activity (MTT-Based Cytotoxicity Assay): The antiparasitic activity of *B.t.* (H14) parasporal inclusion protein was determined by estimating cell viability through the MTT method [11,27]. Briefly, *L. major* promastigotes were washed twice by centrifugation (1000 g for 10 min) with PBS and resuspended in fresh culture medium to a known cell concentration (7×10^7 promastigotes ml^{-1}). To evaluate the parasiticidal activity of *B.t.*, a 100 μl of parasite suspension was incubated in 96-well tissue culture plate for 24h at 24°C with 10 μl of *Bt* 43 kDa protein in a serial final concentration of 100, 50, 25, 12.5, 6.25, 3.12, 1.56 and 0.78 μg ml^{-1} and sterile PBS as control. Each concentration was tested in triplets under similar conditions. Each well then received 30 μl MTT solution [3-(4,5- Dimethyl – 2- thiazolyl) –2,5- diphenyl- 2H-tetrazolium bromide)] (4 mg ml^{-1}) and incubated under dark conditions at 24°C for 4h. At the end of incubation period the medium was removed and the converted dye (reduced formazan) was solubilized by adding 100 μl of acidic isopropanol per well (0.04 - 0.1 N HCl in absolute isopropanol) while shaking for 90-120 min. The parasite viability was determined spectrophotometrically by means of ELISA reader at 540 nm with background subtraction at 630-690 nm. The average absorbance value in buffer inoculated negative control was used as blank value. The arbitrary unit was determined based on the relative value absorbance (optical density at 570 nm) to the blank. The 50% effective concentration (LC$_{50}$) values were deduced from the dose-response curve using log-probit analysis.

RESULTS

Bacillus thuringiensis is a Gram +ve endospore former that releases crystalline δ-endotoxins during vegetation of their spores and can be obtained after lyses of the spores. The obtained spore-crystal complex from *Bt* fermentation was enhanced during the first 12h followed by spontaneous increase in the next 36h then hardly increased in the subsequent 48h (Fig. 1).
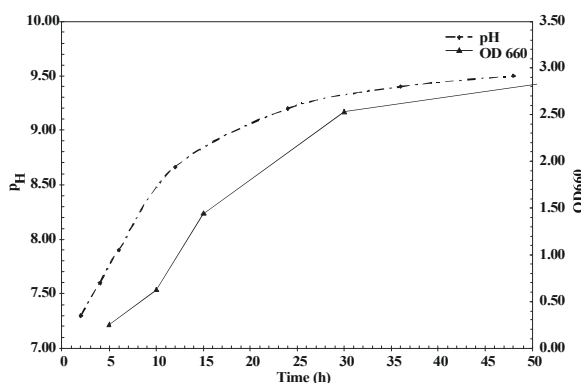

Fig. 1: Growth curve of *Bacillus thuringiensis* in relation to pH of fermentation medium

Table 1: *Bacillus thuringiensis* serovar *thuringiensis* toxic protein recovery during purification process

| Bacterial material	Recovery weight	Protein recovery (%)
Spore crystal complex wet weight after fermentation	16 g/l	-------
Spore crystal complex dry weight after fermentation	2.3 g/l	-------
Starting material after lypholization	7 g	-------
Crystal recovery dry weight	0.1607g/g dry weight	16.07
Dissolved proteins in 1M NaOH pH 12	21.07 mg/g dry weight	2.017
Precipitated toxic protein with 80% NH$_2$SO$_4$	15.784 mg/g dry weight	1.578
Purified protein fraction after molecular weight sieve from 50 – 30 kDa	14.845 mg/g dry weight	1.484
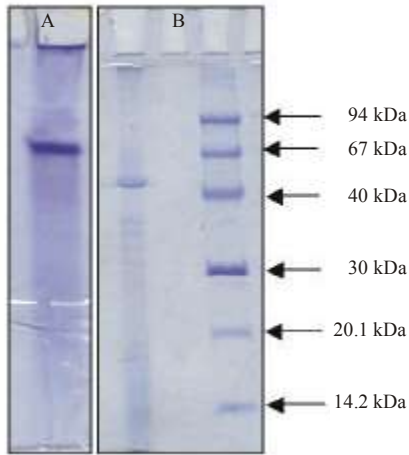
Fig. 2: (A) Native electrophoertic analysis by 7.5% PAGE, (B) 12% SDS-PAGE for *B.t.* seorovar *thuringiensis* toxic protein fraction lane (1) and molecular weight markers lane (2). Molecular weight markers are lysozyme (14.4 kDa), soybean trypsin inhibitor (21.5 kDa), carbonic anhydrase (31 kDa), ovalbumin (43 kDa), bovine serum albumin (66 kDa), phosphorylase b (92 kDa), P-galactosidase (116 kDa) and myosin (200 kDa).

The medium pH played a significant role in diminishing the growth rate where the pH development resulted in reduction of the growth rate (Fig. 1). After 48h, the stationary phase was predominant and the pH markedly increased to 9.5 where sporulation occurred and the crystalline δ-endotoxins was formed.

As shown in Table 1, the obtained spore crystal complex was 16.07 g wet weight L^{-1}. The obtained spore crystal complex was dried by lypholization to 2.31 g L^{-1}. The recovered crystalline protein represented by biphasic separation system was 16.07% of the total dry weight recovered from the fermentation process. The *B.t.* δ-endotoxin crystalline proteins that were dissolved using 1M NaOH pH 12 were completely dissociated by high pH resulting in the untruncated toxic core protein which represented 2.017% of the total dry weight of spore-crystal complex. The acid precipitation of *B.t.* toxic protein followed by ammonium sulfate precipitation using 80% saturated solution diminished the recovered toxic proteins to 1.578%. After application of dialyses and Vivaspine molecular weight concentrator (50 and 30 kDa cutoffs) the recovered toxic proteins constituted 1.484% of total soluble proteins.

The native PAGE revealed that the obtained untruncated toxic protein core is composed mainly of single major intact protein molecule (Fig. 2A). By using 12% SDS-PAGE, it was found that the intact toxic protein fraction is approximately 43 kDa (Fig. 2B).

The bioassay of 43 kDa toxic untruncated *B.t.* serovar *thuringiensis* protein fraction against *L. major* promastigotes revealed that the LC_{50} is 4.95 µg ml^{-1} (Fig. 3).

The cytopathological examination of the treated promastigotes showed that the pathological changes passed through several developmental changes (Fig. 4). The cytopathological changes started with shortening and swelling of promastigotes (Fig. 4A). This was
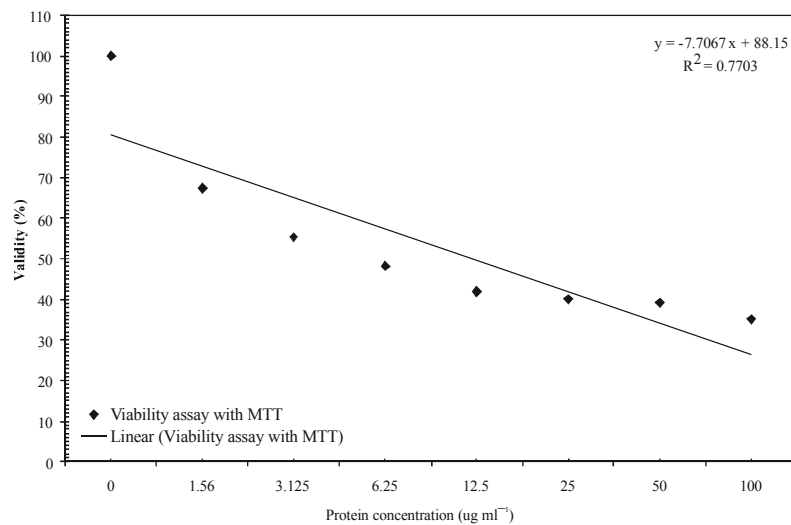

Fig. 3: *Leishmania major* promastigotes viability % versus toxic protein concentration (µg ml^{-1}) after 24h incubation period (LC_{50} = 4.95 µg ml^{-1})
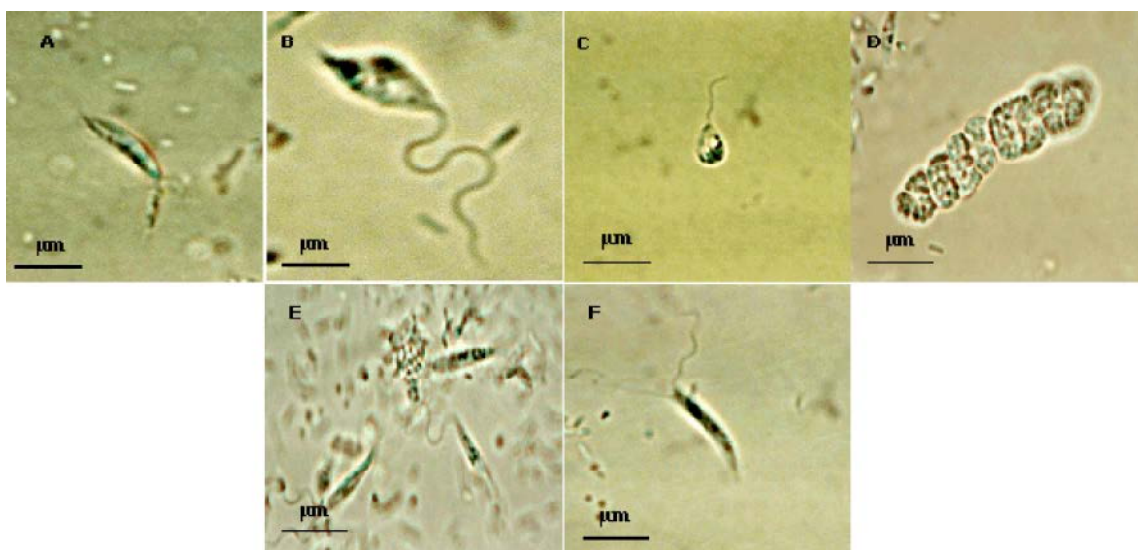
Fig. 4: Cytopathological changes in *Leishmania major* promastigotes started with swelling (A) followed by changing from spindle shape to spheroid (B) berry shaped cells (C) cytoplasmic proteins with a giant increase in size (D). While the untreated promastigotes keep motile and alive in pairs (E&F)

followed by sluggish movement and augmented swelling accompanied by cytoplasmic granulation. The promastigotes changed from spindle shape to spheroid (Fig. 4B) passing through berry shaped cells (Fig. 4C). The cells were then dead and this was followed by complete lyses, loss of flagellae and granulation and segmentation of the cytoplasmic proteins with a giant increase in size (Fig. 4D). In the control group however, the untreated promastigotes were observed motile and alive in pairs (Fig. 4E &F).

DISCUSSION

The growth curve of *Bacillus thuringiensis* versus pH revealed that there is a direct relationship between the growth rate and the media pH. The stationary phase was predominant after 48h and the pH markedly increased to 9.5 where cell lyses and sporulation occurred and the crystalline δ-endotoxins were formed. These results corroborate those previously recorded by Gad El-Said *et al.* [28] and Shi *et al.* [29]. The recovery percentage of untruncated toxic core by pH hydrolysis constitutes about 1.484% of the total dissolved proteins which is sufficient as biological active material. The toxic untruncated core protein is about 43 kDa which is already reported by other investigators working with *Bt* parasporal inclusion proteins from other serovars [30,31]. There are, as yet, no reports available regarding the activity of *Bt* delta endotoxins against *Leishmania* sp. In

the present study, the toxic protein fraction induced cytotoxicity to promastigotes at the LC_{50} of 4.95 µg ml^{-1}. This resulted in developmental cytopathological changes which started with swelling of promastigotes. This swelling is considered as an ordinary symptom for toxicity of parasitic cells with *B.t.* delta endotoxins [32]. However, degenerative changes subsequently occurred in drastic manner including cytoplasmic granulation, cytoplasmic lobulation, loss of flagellae and complete lyses after 24h of treatment. The promastigotes showed some resistance for cytotoxicity represented in melanin production which can be noticed as granulation of the cells in early stages of cytotoxicity, a mechanism that might be similar to that observed in filarial nematodes [33]. This defense mechanism was not successful as the *Leishmania* promastigotes were completely deformed. According to the cytopathological changes observed in the present investigation, several possible explanations are available. One explanation would be due to the effect of the cytotoxic protein on the cell membrane and cytoplasmic proteins of *Leishmania* promastigotes. The size of the promastigotes increased prominently (ten times or more) which could be due to loss of cell membrane permeability control through binding of 43kDa protein Cry4Ba domain with specific cell receptor [34]. While the cytoplasmic matter showed marked granulation or agglutination, this coul be referred to agglutination of cytoplasmic proteins due to ionic disturbance as a result of induced Ca^{+2} influx and a sustained elevation of the cytoplasmic

Ca^{+2} concentration in toxin-sensitive cells [35]. The above mentioned explanations are partially established but the mode of action of *B.t.* cytotoxic protein remains unclear.

This study has shown that *B.t.* delta endotoxin is effective against *Leishmania* promastigotes. However, the mechanism behind its antileishmanial effect needs to be fully understood. The susceptibility of *Leishmania* to this protein toxin *in vivo* needs to be assessed. Moreover, studies involving the effect of *B.t.* crystal protein on *Leishmania*-infected sand flies are also needed. These studies could ultimately lead to the introduction of *B.t.* as a safe therapeutic antileishmanial agent.

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