# SELECTIVE CYTOTOXIC ACTIVITY AGAINST LEUKEMIC CELL LINES FROM MOSQUITOCIDAL *BACILLUS THURINGIENSIS* PARASPORAL INCLUSIONS

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Abstract. The discovery of parasporin has triggered an interest in examining various *Bacillus thuringiensis* (Bt) isolates for specific anti-cancer activity. The aim of this study was to determine the potency and specificity of parasporal inclusions from Malaysian mosquitocidal Bt isolates against a leukemic cell line (CEM-SS). The Bt isolates used in this study were identified as having weak to potent larvicidal activity against *Aedes aegypti* and varying hemolytic activity. The 12 mosquitocidal Bt isolates examined in this study showed low to moderate cytotoxicity when tested against CEM-SS and HeLa. Interestingly the parasporal inclusions of Bt 18 (non-hemolytic isolate), showed therapeutic potential demonstrating specificity for CEM-SS compared to HeLa, whilst being non-cytotoxic to normal T lymphocytes. The mode of cell death by Bt 18 was shown to be initially apoptotic. SDS-PAGE analysis and N-terminal sequencing of the upper and lower bands of Bt 18 showed similarity between Bt 18 parasporal inclusions with Cry 24Aa and 25Aa of *Bacillus thuringiensis* subsp *jegathesan* and Cry 15Aa of *Bacillus thuringiensis* subsp *israelensis*. Although the BLAST analysis did not show sequence similarity between Bt 18 and Parasporin, we propose that the Bt 18 parasporal inclusions share similar characteristics to Parasporin since Bt 18 is not hemolytic but discriminately cytotoxic towards leukemic cell lines.

## INTRODUCTION

Insecticidal *Bacillus thuringiensis* (Bt) produces parasporal inclusions (proteins) known as δ-endotoxin. These parasporal inclusions are encoded by a family of *cry* and *cyt* genes (Hofte and Whiteley, 1989) and have been successfully used as a biological control agents for decades. Recently, a study of non-insecticidal Bt parasporal inclusions suggested the presence of a new family of parasporal inclusions designated as parasporin (PS) with cytotoxic activity agaist leukemic T-cells (Mizuki *et al,* 2000). The proteolytically activated PS showed strong cytotoxic activity against leukemic T-cells (MOLT-4) and human uterus cervix cancer cells (HeLa) but not to normal T- cells (Mizuki *et al,* 1999, 2000). The amino acid sequence of PS contains the 5 conserved blocks commonly found in Bt Cry proteins but with low homologies (<25%). Hence, there have been suggestions that the mode of action of PS might be similar to that of Cry toxins, which requires solubilization, activation with proteases and binding to glycoprotein receptors prior to pore formation in the target cells (Lee *et al,* 2001). PS has since been classified as a non-hemolytic Bt parasporal inclusion protein which can preferentially kill cancer cell lines. There are currently four subclasses of PS (PS1 to PS4) and a nomenclature and classification committee

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for PS has been established (Saitoh *et al,* 2006).

The Bt isolates used in this study were from the Institute for Medical Research (IMR) collection and have been identified as having weak to potent larvicidal activity against *Aedes aegypti* and selective hemolytic activity (Nadarajah *et al,* 2006). The Malaysian soil has been a rich source of potent and commercially viable mosquitocidal Bt isolates, for example *Bacillus thuringiensis* subsp *malaysianiensis* and *Bacillus thuringiensis* subsp *jegathesan* (Btj). The diversity of Malaysian isolates expands with the discovery of the *cry9a* gene (a member of the Bt Cry family of genes), which encodes the insecticidal Cry9A toxins in *Clostridium bifermentans*, also isolated from Malaysian soil (de Barjac *et al,* 1990; Lee and Seleena, 1990). The diversity in flagellar H-antigen agglutination reactions observed from the Malaysian soil is also an indication of genetic diversity among Bt isolates. Furthermore, the discovery of PS has triggered an interest in examining various Bt isolates with specific anti-cancer activity. Hence, the aim of this study was to determine the potency and specificity of parasporal inclusions from Malaysian Bt isolates against leukemic cell lines. The hypothesis of this study was that these Bt isolates would produce parasporal inclusions with specific anticancer activity as suggested by their diverse bioactivity.

This investigation shows that the mosquitocidal Bt isolates had varied activity against the cancer cell line studied, with one isolate identified to have therapeutic potential.

## MATERIALS AND METHODS

### Bacterial strains and growth condition

The Bt isolates used in this study were designated as Bt 1, 2, 4, 7, 8, 9, 10, 18, 19, 20; j and IPS 82 were from the IMR Bt collections. The Bt subtypes were determined using H serotyping by the Institute Pasteur, Paris and were found to be either *Bacillus thuringiensis* subsp *israelensis* (Bti), Btj or *Bacillus thuringiensis* subsp *morrisoni* (Btm). These isolates were grown on nutrient agar, pH 7 and 30ºC until sporulation was completed (approximately 48-72 hours). A loopful of sporulated Bt isolate was placed into 0.5 ml sterile deionized water ($dH_20$), vortexed vigorously to disperse clumps and heated in waterbath at 75ºC for 30 minutes to kill vegetative and activate spores for germination. The 0.5 ml activated spore aliquot was then transferred to 250 ml of prewarmed and preaerated nutrient broth medium at pH 7. This culture was incubated at 30ºC with constant shaking at 250 rpm until greater than 95% free phase-bright spores were produced (approximately 48 hours).

### Preparation of spore-crystal mixtures from cultures of Bt isolates

Solid NaCl was added to sporulated nutrient broth cultures of Bt to a concentration of 1M to induce lysis. The cultures were immediately shaken and cooled on ice. The cultures were centrifuged at 6,000*g* for 10 minutes at 4ºC and the resultant spore-crystal pellet was washed once with 1M NaCl, twice with ice-cold $dH_20$ and resuspended in an appropriate volume of Tris/KCl buffer (50 mM Tris/HCl, 10 mM KCl, pH 7.5). The spore-crystal mixture was aliquoted and stored at -20ºC until further use.

### Solubilization and activation of parasporal inclusion proteins

Parasporal inclusion proteins were harvested from cultures by solubilizing the spore-crystal mixture in 50mM $Na_2CO_3$, 10 mM DTT, pH 10.5, for one hour (except for solubilization studies, various pH were used to solubilize the parasporal inclusion to determine the optimum pH for solubilization). The insoluble spore and other debris were sedimented by centrifugation at 13,000*g* for 5 minutes. The resultant pellet was discarded and the supernatant (solubilized parasporal inclusion) was then activated with proteolytic enzymes, 1 mg/ml

of trypsin at the ratio of 1:10 proteolytic enzymes to parasporal inclusions for one hour at 37ºC. This was followed by further centrifugation at 13,000*g* for 5 minutes and the resultant supernatant was designated as solubilized and activated parasporal inclusion.

SDS-PAGE analysis

The samples were loaded onto a 10% SDS-PAGE gel after treatment with the gel sample buffer for 5 minutes at 95ºC. Electrophoresis was performed at 150V for 45 minutes. After electrophoresis, the gels were stained with Coomassie blue. The molecular masses of the proteins were estimated by comparison to molecular weight standards (Bio-Rad).

Cancer cells lines and culture conditions

Four human cell lines, CEM-SS (human leukemic T cell), HeLa (human uterus cervix cancer cell), MCF 7 (human breast carcinoma cells) and HT-29 (colorectal cancer cell) were cultured in either RPMI 1640 medium (CEM-SS) or DMEM (HeLa, MCF 7, HT-29), supplemented with 10% fetal bovine serum and 100 IU/ml penicillin and 100 μg/ml streptomycin (Sigma, USA) at 37ºC in humidified 5% $CO_2$.

Cytotoxic assay

The pH of the solubilized and activated parasporal inclusions was adjusted to 7.4 and the inclusions were filtered using a 0.2 μm syringe filter. The concentration of the inclusion protein was then determined using the Bradford method. In each well of the 96 well plate, 100 μl of cells, at a density of 1 x $10^6$ cells/ml for CEM-SS and 1 x $10^5$ cells/ml HeLa, was incubated with 100 μl of protein at different concentrations for 72 hours at 37ºC. The level of cytotoxicity of activated inclusion protein was assessed with the MTT assay, a cell proliferation assay (Heiss *et al,* 1997). The cytotoxic activity of the inclusion protein was determined by comparing the percentage of cell killing of the treated cell population with the untreated control population, indicated by their respective absorbance.

Morphological changes

CEM-SS cells (1 x $10^6$ cells/ml) were exposed to solubilized and activated Bt 18 parasporal inclusions at 0.5 mg/ml (highest concentration tested) for 72 hours and the morphological changes were determined using acridine orange (AO) and propidium iodide (PI) staining. Cell suspensions were mixed with an equal volume of staining solution containing 10 μg/ml AO and 10 μg/ml PI (dissolved in PBS) and observed under a fluorescent microscope within 30 minutes.

Fast phase liquid chromatography purification of parasporal inclusion proteins

The solubilized, activated and desalted Bt 18 parasporal inclusion protein was loaded (1 ml) into the RESOURCE Q anion exchange column (Amersham Biosciences, Sweden) attached to a FPLC system. The column was equilibrated with 20 mM Piperazine at pH 9.8. The parasporal inclusions were then eluted from the column with a linear salt gradient, 0 to 1M NaCl, monitored at 280 nm. Fractions of peaks were collected for further analysis.

N-terminal sequencing

The solubilized and activated Bt 18 samples were loaded on SDS-PAGE gel, and run under conditions as described earlier in the SDS-PAGE analysis section. The difference for the N-terminal sequencing was that the gel was not stained with Coomassie blue, but was instead used as a template for transfer onto a wet PVDF membrane using cold CAPS (3-[cyclohexylamino]-1-propanesulfonic acid) buffer. The electrotransfer was performed at 85 volts for one hour. The transferred proteins were stained by Coomassie blue R-250 (0.1 w/v) before being sent for N-terminal sequencing (First BASE Laboratories).

## RESULTS

Solubilization and activation of the parasporal inclusions

The study began when sporulated Bt

cultures were obtained after the Bt strains were grown on nutrient agar, pH 7 at 30ºC for 3 days. The spore-crystal mixture was prepared from the sporulated cultures as described in the methods section. Solubilization in appropriate buffers at optimum pH is necessary to separate the parasporal inclusions from the spore-crystal mixture. To determine the optimum pH for solubilization of the Bt parasporal inclusions, each Bt spore-crystal mixture was solubilized in 50 mM $Na_2CO_3$, with 10 mM DTT at 7 different pH values, pH 9-12. The protein concentration of the spore-crystal mixture was determined, using the Bradford reagent before and after solubilization to calculate the percentage of protein recovery (Table 1 shows examples of solubilization studies on Bt 18 and Bt 20). Our data indicates that for all Bt strains used in this study, pH 10.5, yielded the highest protein recovery and was selected as the optimum pH for further studies.

Cytotoxic assay

To examine the cytopathic effects of the parasporal inclusions on cancer cell lines, the solubilized and activated parasporal inclusions were tested against CEM-SS and HeLa cell lines. As shown in Table 2, cytotoxic activity against CEM-SS was observed for Bt 1, 2, 4, 7, 8, 9, 10, 18, 19, 20, j and IPS 82 when tested at the maximum concentration of 0.5 μg/ml, respectively. Among the isolates tested, Bt 18 was found to be the most cytotoxic against CEM-SS, and Bt 1 was the most cytotoxic against HeLa. Bt18 and Bt 8 demonstrated selectivity because it showed high cytotoxic activity against CEM-SS but minimal cytotoxic activity against HeLa. Fig 1 shows the dose-response curve of percentage cell viability versus toxin concentrations for CEM-SS and HeLa after treatment with Bt 1, Bt 8 and Bt 18. The cytotoxicity of Bt 1, Bt 8 and Bt 18, is dependent upon the amount of toxin and the type of cell line. The $CD_{50}$ of Bt1, Bt8 and Bt18 against HeLa and CEM-SS are summarized in Table 3; the $CD_{50}$ values confirm Bt18 as most toxic to CEM-SS and Bt 1 as most toxic to HeLa. Importantly the $CD_{50}$ values suggest selectivity of Bt 8 and Bt 18 for CEM-SS compared to HeLa. Interestingly when Bt 18 was further examined for activity against MCF 7 and HT 29, cytopathic activity was not observed when concentrations were increased to a maximum of 0.5 mg/ml.

Morphological studies

To determine the mode of cell killing, Bt 18 treated CEM-SS was stained with acridine

Table 1
Recovery of Bt 8 and Bt 18 parasporal inclusions after solubilization.

| | pH | | | | | | |
|---|---|---|---|---|---|---|---|
| | 9 | 9.5 | 10 | 10.5 | 11 | 11.5 | 12 |
| [Bt 8]μg/ml | 94 | 93 | 93.5 | 117 | 75 | 80 | 77.5 |
| % recovery | 25.6 | 25.4 | 25.6 | 32.4 | 20.5 | 21.8 | 21.3 |
| [Bt 18]μg/ml | 65.8 | 64.2 | 61 | 106 | 42 | 44 | 40 |
| % recovery | 10 | 9.76 | 9.3 | 16.1 | 6.4 | 6.7 | 6.0 |

The toxins were solubilized in alkaline conditions ranging from pH 9 to pH 12 with 50 mM of $Na_2CO_3$, 10 mM of DTT for one hour, at 37ºC. Bio-Rad protein assay kit with BSA as the standard was used to determine the concentration of toxin. Protein concentrations before solubilization for Bt 8 and Bt 18 were 361.11μg/ml and 658.39 μg/ml, respectively. Absorbance was taken at 595 nm. To calculate the percentage of protein recovery the following formula is used,

$$\frac{\text{Protein concentration after solubilization}}{\text{Protein concentration before solubilization}} \times 100$$

orange and propidium iodide in a time course assay. The cells that had nuclei stained with bright yellow-green fluorescence. Those with diffuse chromatin was counted as viable cells and those with condensed fragmented nuclei were considered to be apoptotic cells. Cells with nuclei stained orange-red with no chromatin condensation were considered as necrotic cells. Fig 2 shows acridine orange and propidium iodide stained CEM-SS after being treated with Bt 18. At 0 hour, there were mainly green intact viable cells (Fig 1a). At 24 hours, there were a significant numbers of apoptotic cells (Fig 2b). The clumped cells were also found to exhibit necrotic cellular lesions, which is normally termed coagulation necrosis. This phenomenon was more apparent at 48 and 72 hours of treatment, while apoptotic cells were found less often (Fig 2c and 2d). There were increasing numbers of necrotic cells over the 72 hour treatment period.

Table 2
Cytotoxic activity of Bt parasporal inclusions on cancer cell lines.

| Toxin | Cytotoxic activity (percentage of lysed cells) | |
|---|---|---|
| | CEM-SS | HeLa |
| Bt 1 | 30 | 85 |
| Bt 2 | 52 | 34 |
| Bt 4 | 43 | 45 |
| Bt 7 | 38 | 12 |
| Bt 8 | 56 | 5 |
| Bt 9 | 43 | 20 |
| Bt 10 | 69 | 51 |
| Bt 18 | 84 | 27 |
| Bt 19 | 15 | 13 |
| Bt 20 | 10 | 23 |
| IPS 82 | 51 | 50 |
| Btj | 77 | 71 |

The table shows the mean percentages of cell lysis at 0.5 μg/ml (the highest concentration tested) of Bt toxins compared relatively to the control untreated cell population. Cells were incubated in triplicate with solubilized and activated Bt toxins from various isolates for 72 hours and subjected to MTT assay for determination of cell viability. The standard deviation for all the mean values presented was less than 0.5.

Purification of Bt 18 parasporal inclusions and N-terminal sequencing

As Bt 18 showed cytopathic selectivity, it was selected for further investigation. SDS-PAGE analysis of solubilized and activated Bt 18 showed a major upper band, a doublet of approximately 60 to 64 kDa and lower band of 28 kDa (Fig 3). To determine the cytotoxic activity of the upper and lower bands the solubilized and activated Bt 18 were subjected to anion exchange chromatography analysis using a FPLC system, with the aim to obtain separate fractions of the upper and lower bands. After the FPLC anion exchange at a 30% salt gradient, a major peak was observed in the elution curve and the SDS-PAGE analysis of this peak shows a preparation of Bt 18

Table 3
Toxicities measured by $CD_{50}$ values, for Bt1 Bt8 and Bt18 against CEM-SS and Hela.

| Toxin | Bt 1 $CD_{50}$ value (μg/ml) | Bt 8 $CD_{50}$ values (μg/ml) | Bt 18 $CD_{50}$ values (μg/ml) |
|---|---|---|---|
| Cell lines | | | |
| CEM-SS | 0.2375±0.0045 | 0.2178±0.0032 | 0.1224±0.0092 |
| HeLa | 0.0323±0.0025 | >0.5 | >0.5 |

The toxicities of Bt1, Bt8 and Bt18 against CEM-SS and HeLa were measured in terms of the concentrations that killed 50% of the cells ($CD_{50}$) compared to the untreated control cell population. The $CD_{50}$ values were determined using Biograph analysis software from the University of Strachlyde,UK.

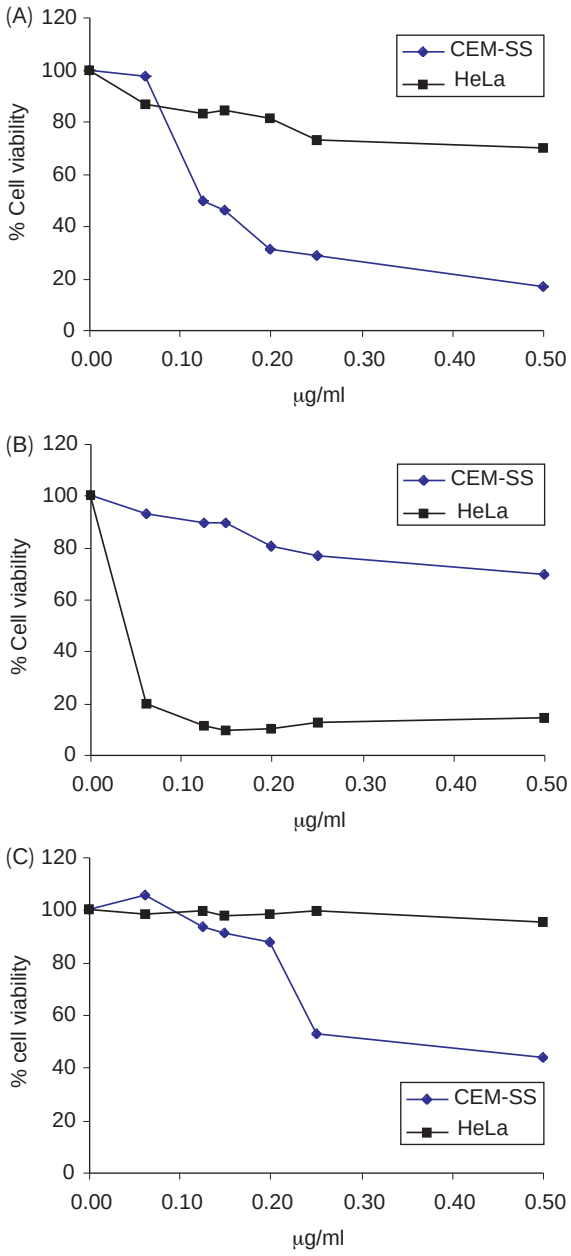



Fig 1–The dose response curve of percentage of cell viability versus toxin concentrations for CEM-SS and HeLa after treatment with different concentrations of Bt 18(A), Bt 1(B) and Bt 8 (C). The highest concentration tested was 0.5 μg/ml followed by 0.25, 0.2, 0.15, 0.125 and 0.0625μg/ml. Data shown were deduced from the mean of the triplicate absorbance reading determined using the MTT assay.

with the upper band only remaining (Fig 3). This purified Bt 18 inclusion was examined for cytotoxic activity against CEM-SS cell lines and cytotoxicity was observed to be approximately 20% lower than that of the non-purified samples.

To identify the cytotoxic polypeptides of Bt 18, the solubilized and activated Bt 18 upper and lower bands were subjected to N-terminal sequencing. The N-terminal sequence of the upper band (60 kDa) was DGNYKDLLNE whilst the lower band (28 kDa) sequence was TITNIELAIRD. When both the sequences were analyzed using the NIH BLAST Program (http://www.ncbi.nlm.nih.gov/BLAST), it was observed that the sequence similarity for the upper band was best with Cry24Aa and Cry25Aa isolated from Btj. For the lower band sequence similarity was best with Cry15Aa of Bti ATCC 35646.

## DISCUSSION

In this study, cytotoxic activity of various mosquitocidal Malaysian Bt isolates were investigated. These isolates, were either subtypes of Bti, Btm or Btj, and found to exhibit varied toxicity against mosquito larvae (Lee HL, unpublished data). Studies have shown the high toxicity of Btm parasporal bodies to mosquito larvae is due to the presence of similar proteins responsible for the high toxicity of Bti (Guerchichoff *et al,* 2001). Therefore, it is assumed that these Bt isolates collected by the Institute for Medical Research (IMR) although of differing subtypes may produce parasporal inclusions proteins that cause them to be larvicidal.

To determine the cytotoxic activity of these Bt isolates, the parasporal inclusions need to be extracted from the spore crystal mixture, hence solubilization in an alkaline buffer was necessary. The data indicate that for all Bt isolates used in this study, a pH of 10.5 yielded the highest protein recovery. The results of the

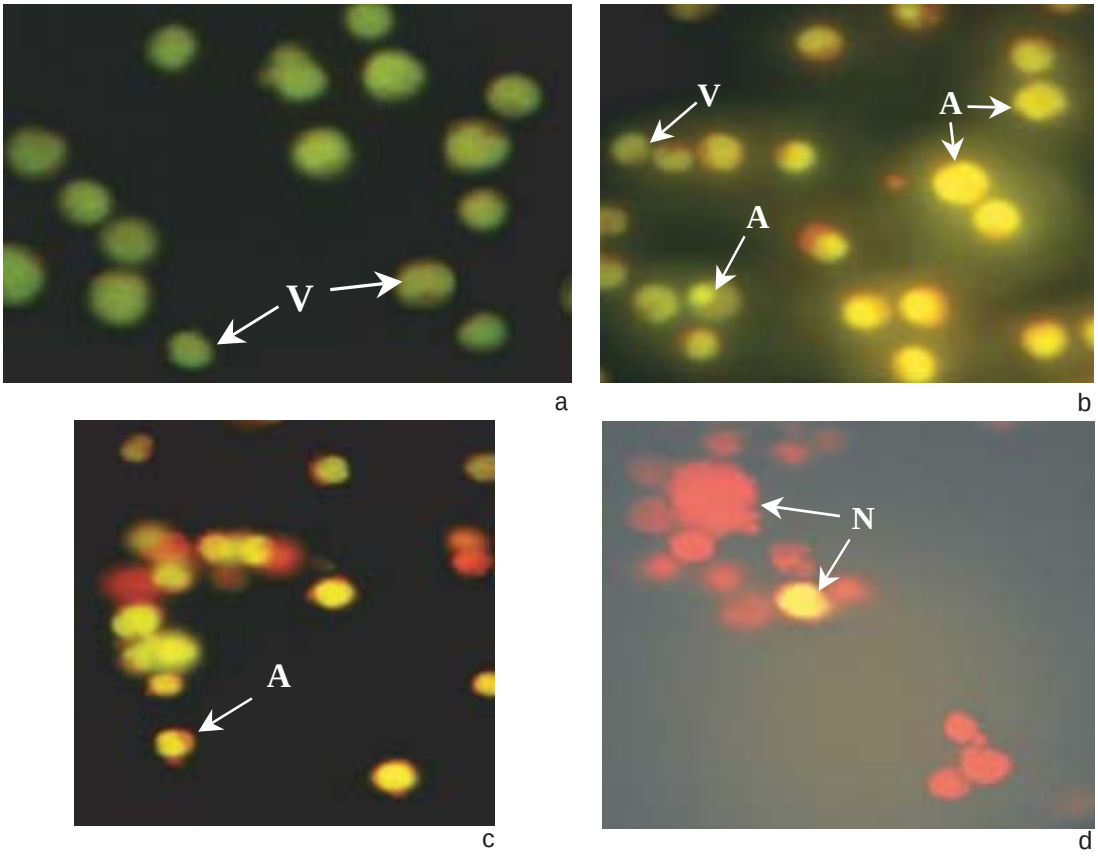
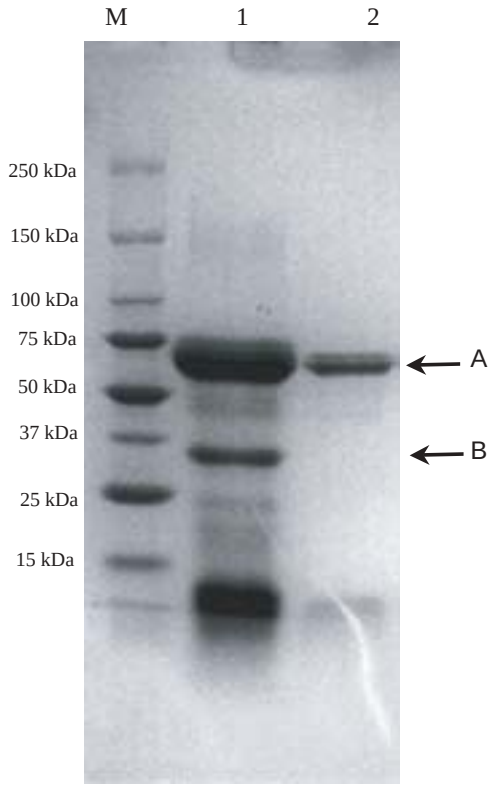



Fig 2–Photomicrographs of acridine orange-propidium iodide stained CEM-SS cells. Treatment of CEM-SS with Bt 18 at (a) 0 hours, (b) 24 hours, (c) 48 hours and (d) 72 hours. V = viable cells; A = apoptotic cells and N = primary or secondary apoptosis. Magnification 200x.

solubilization assay are supported by the fact that the environment of the mosquitocidal gut is alkaline. Different Bt subspecies, for example *shandongiensis* (Lee *et al,* 2001) and *coreanensis* (Namba *et al,* 2003) were solubilized at an alkaline pH to yield an optimum amount of toxin with cytotoxic activity.

Studies have also shown that a 25 kDa protein present in Bti is cytotoxic to cultured tumor cells in mice and can also potentiate the cytotoxic effects of some anti-tumor agents *in vitro,* for example bleomycin (Yokoyama *et al,* 1991). In 2000, when Bti was tested with human leukemic cells (MOLT-4), uterus cervix cancer cells (HeLa) and normal T cells, Bti exhibited indiscriminative toxicity against all of the three cells (Yamashita *et al,* 2000). It was shown that the Cyt1 toxin, a broad-spectrum cytolysin was contained in the parasporal inclusions of the Bti and was responsible for the cytotoxic properties (Yamashita *et al,* 2000). The potent mosquitocidal activity observed in Bti has been attributed to the presence of Cyt1.

In this study, 12 Bt isolates collected from IMR were tested against a type of human leukemic T cell, CEM-SS, to elucidate cytotoxicity. Generally, all the isolates tested demonstrated low to high levels of cytotoxicity against CEM-SS. As mentioned earlier, most of these isolates were found to be subtypes of Bti. Hence, it is suggested that the cytotoxic properties found in some of the Bt isolates may be




A: Upper band 60 kDa (lane 2)
B: Lower band 28 kDa (lane 1)

Fig 3–SDS-PAGE analysis of Bt 18 parasporal inclusions. The analysis was performed on 10% polyacrylamide gel and was stained with Coomassie blue R-250. Lane M: Precision Plus Protein Standards (Bio-Rad, California, USA). Lane 1: Solubilized and activated parasporal inclusions of Bt 18. Lane 2: Solubilized, activated and purified parasporal inclusion of Bt 18.

due to the presence of Cyt1 toxin.

Table 2 shows that Bt 18 demonstrated the highest cytotoxic activity against CEM-SS and the lowest cytotoxicity against HeLa. Bt 8 also demonstrated selective cytotoxicity against CEM-SS and Bt 1 was found to be most cytotoxic against HeLa. It is interesting to note that both Bt 8 and Bt 1 are subtypes of Bti, yet they had very different cytotoxic profiles. Bt 18 on the other hand is a subtype of Btm. It has been well established that the insecticidal proteins of *Bacillus thuringiensis* discriminate between susceptible and non-susceptible cells by the presence or absence of specific receptor proteins in the cell membrane (Schnepf *et al,* 1998). The results of this study suggest that CEM-SS and HeLa may have particular molecular receptor site (s) with which Bt isolates react effectively to produce a strong toxic response.

There have been recent reports of Bt parasporal inclusions with nonhemolytic activity by selective anticancer cytopathic activity. This has led to the discovery of Cry31Aa, Cry46Aa, Cry41Aa and Cry45Aa, now designated as a group of toxins called PS (Mizuki *et al,* 2000; Ito *et al,* 2004; Yamashita *et al,* 2005; Saitoh *et al,* 2006). In a previous study of these Malaysian Bt isolates (Nadarajah *et al,* 2006) it was observed that trypsin activated Bt 8 and Bt 18 had non-hemolytic activity against rat and human erythrocytes. In an attempt to elucidate the therapeutic potential of Bt 8 and Bt 18, the $CD_{50}$ levels of these inclusions was calculated against CEM-SS and HeLa. Bt 18 showed a high level of toxic activity, killing 50% of the cells at a concentration of 0.12 μg/ml. However, it should be noted the $CD_{50}$ of some PS towards other types of cancer cell lines have been reported to be lower than the values observed in this study (Kitada *et al,* 2006; Saitoh *et al,* 2006).

Previous studies showed that the cytopathological changes of Bt treated cancer cells were characterized by condensation of the nucleus, cell-ballooning and mitochondrial swelling associated with apoptosis (Mizuki *et al,* 2000; Yamashita *et al,* 2000; Lee *et al,* 2001). Recently, a study of the cytotoxicity effects *Bacillus thuringiensis* Bt subsp *coreanensis* was carried out against MOLT-4, observation of the cells stained with PI and Annexin V-FITC under fluorescent microscopy suggested that the proteinase-K activated crystal proteins induced the apoptotic cell

death of MOLT-4 (Namba *et al,* 2003). Morphological analysis of Bt 18 induced cell death on CEM-SS has been assessed at the level of light and fluorescent microscopy. Assessment under normal light microscopy indicates that both apoptotic and necrotic death occurred simultaneously in the same population following treatment with Bt 18. The biochemical involvement is not known at this stage and investigations are ongoing. The presence of both apoptotic and necrotic cells in Bt 18 was further identified using dual fluorescent staining, acridine orange (AO) and propidium iodide (PI). Apoptotic death was more prominent at earlier periods of treatment, within 24 hours. Necrotic death was detected during the later period of incubation. It is possible that cells that have undergone apoptosis have progressed into secondary necrosis due to prolonged incubation with the toxin.

When Bt 18 was tested against normal T lymphocytes, no cytotoxic effects were observed even at the highest concentration of 0.5mg/ml (Chan *et al,* 2007). Considering the non-hemolytic and discriminative cytotoxic properties of Bt 18, it was of interest to try and identify the parasporal inclusions of Bt 18. As for Bt 1 and Bt 8, although there was significant toxicity against human cancer cells, they also demonstrated cytotoxicity against normal T lymphocytes, an indiscriminative property which is harmful to human beings.

SDS-PAGE analysis showed that solubilized and activated Bt 18 contains a lower band of 28 kDa polypeptide which is of a similar molecular weight to the protein expressed by *Bacillus thuringiensis* subsp *shandongiensis,* a Bt strain that is found to be discriminative in its cytotoxic effects (Lee *et al,* 2001). SDS-PAGE analysis also showed an upper band which may be a doublet of approximately 60 to 64 kDa, a molecular weight close to Parasporin 1 (56 kDa) (Katayama *et al,* 2005). To determine whether the upper band or lower band had cytotoxic activity, anion exchange chromatography was applied, and only the upper band was eluted. This eluted fraction maintained discriminative cytotoxic activity against CEM-SS. Attempts to isolate the 28 kDa protein are ongoing.

Although Btj, Bti and Bt18 (a subtype of Btm) are of different serotypes, the N-terminal sequencing data of the upper and lower bands indicates that Bt18 has produced parasporal inclusions with a high sequence homology with Btj and Bti. This is not surprising, since Btj, Bti and Btm are known for their potent mosquitocidal activity (Chang *et al,* 1992; Kawalek *et al,* 1995) and may share similar Cry or Cyt toxins that contribute to mosquitocidal activity. Bt 18 also has weak mosquitocidal activity (Lee HL, unpublished data.) A comparison of the SDS–PAGE profile of Bt 18 with solubilized and activated parasporal inclusions of Bti and Btj (Nadarajah *et al,* 2006) shows the Bt 18 polypeptide profile to be different. Balaraman (2005) states that 36 subspecies toxic to mosquitoes have been reported worldwide from different sources, such as soil (including mangrove), water, animal feces and plants. The difference in bio-toxicity between isolates may be due to the presence or absence of *cry* or *cyt* genes caused by the loss of plasmids that harbor these genes. Bt 18, although identified as serovar of *morrisoni*, produces parasporal inclusions with specific cytotoxic activity against leukemic cells, which may be due to the environment it was isolated from or changes in the genetics of the parasporal inclusions it produces.

In conclusion, although blast analysis did not show sequence similarity between the Bt 18 polypeptides with Parasporin, we propose that the solubilized and trypsin activated Bt 18 polypeptide shares similar characteristics with Parasporin since it is not hemolytic but discriminately cytotoxic towards leukemic cell lines. This finding warrants further investigation, including cloning the specific genes for

the parasporal inclusions and elucidating the mechanism of action.

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