Original Research

Journal Home page : www.jeb.co.in ★ E-mail : editor@jeb.co.in

Journal of Environmental Biology

DOI : http://doi.org/10.22438/jeb/39/6/MRN-725

JEB™

p-ISSN: 0254-8704
e-ISSN: 2394-0379
CODEN: JEBIDP


# Characterization of native *Bacillus thuringiensis* strains for cytotoxicity against human cancer cell lines



## Authors Info

**M.K. Oktay, B. Şahin and H. Güneş***

Department of Biology, Faculty of Science, Muğla Sıtkı Koçman University, 48000, Muğla, Turkey

*Corresponding Author Email : haticegunes@mu.edu.tr

## Key words

*Bacillus thuringiensis*
Cancer cell lines
*Cyt* genes
Cytotoxicity
Parasporin

## Publication Info

Paper received : 14.08.2017
Revised received : 20.12.2017
Accepted : 05.02.2018

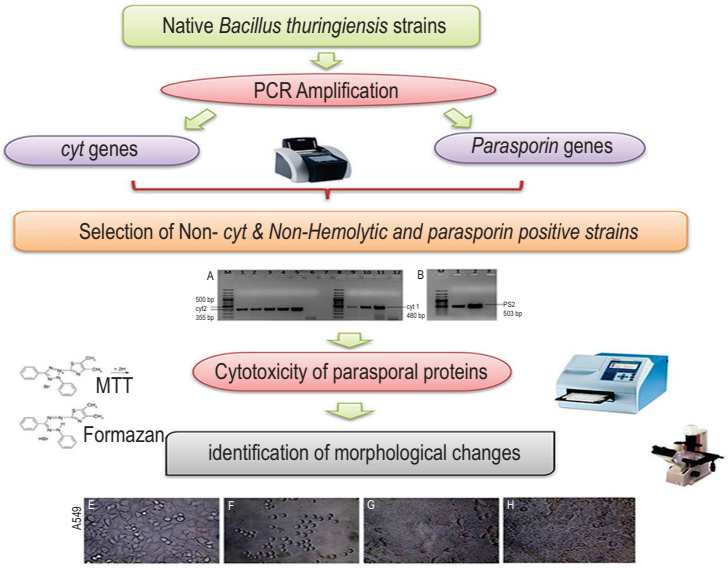
## Abstract

**Aim :** The aim of this study was to characterize fourteen *B. thuringiensis* strains for *cyt* and *parasporin* gene contents as well as cytotoxicity of parasporal proteins against different cancer cell lines.

**Methodology :** PCR was applied for screening of *cyt 1/cyt 2* and six different *parasporin* genes. In addition, *cyt* negative (non-*cyt*) nine Bt strains were tested for hemolytic activity. Non-cyt and non-hemolytic parasporal proteins from nine Bt strains were alkali solubilized and activated by proteinase- K for in *vitro* cytotoxic activities.

**Results :** Five Bt strains carried cyt gene whereas only one strain Bt-Ba14 harbored *parasporin 2* gene and showed 37 kDa protein on SDS-PAGE. Non-*cyt* 9 Bt strains were confirmed to be non-hemolytic. MTT assay indicated that activated parasporal proteins (10 µg $ml^{-1}$) displayed selective cytotoxicity against HeLa, PC-3 and A549 cancer cell lines compared to a normal cell line BEAS-2B. Parasporal protein from Bt-Ba14 exhibited the highest cytotoxicity against all three cancer cells, however, not to normal cell line and caused cytocidal activity similar to that observed with parasporins.

**Interpretation :** The study reported that non-cyt and non-hemolytic Bt strains from Turkey have parasporal proteins with cytotoxic activities against cancer cells.



## Introduction

*Bacillus thuringiensis* is a Gram positive, entomopathogenic and spore forming bacterium that produce parasporal inclusions during sporulation (Roh *et al*., 2007; Höfte and Whiteley, 1989). The parasporal crystalline proteins are made of delta-endotoxins and divided into two classes, Cry and Cyt proteins (Guerchicoff *et al*., 2001). The Cry protein has strong larvicidal activity specific for Lepidoptera, Coleoptera and Diptera (Schnepf *et al*., 1998), whereas Cyt protein has a wide range of cytolytic and hemolytic activities aganist different invertebrate and vertebrate cells (Crickmore *et al*., 1998; Palma *et al*., 2014).

Cry proteins exhibit their effects in the midgut epithelial cells of susceptible insect after certain processes including solubilization of the inclusions under alkaline conditions, proteolytic cleavage of protoxin by midgut proteases, binding to specific receptors located on the epithelial cell surface, and causing the pore formation that leads to cell lysis (Schnepf *et al.,* 1998). Toxicity of Cyt toxin is not sufficient by itself; however, its insecticidal activity is mediated by synergistic interaction with the Cry proteins (Salehi *et al.,* 2008; Soberon *et al.,* 2012).

During last decades, more than 700 cry genes coding for Cry proteins have been determined (Palma, 2014). Even though many Cry proteins have insecticidal activity, some of them have toxicity against bacteria, nematodes, parasites and human cancer cells. In fact, non-insecticidal *B. thuringiensis* strains are more widely distributed than insecticidal strains in natural environments (Ohba, 2000; Mizuki *et al.,* 1999). The biological role of these strains were unknown until Mizuki *et al.* (1999) had identified their function. They reported a unique cytocidal activity from non-insecticidal and non-hemolytic *B. thuringiensis* strains aganist human cancer cells. Bioactive molecule from these *B. thuringiensis* strains is called parasporin (Mizuki *et al.,* 2000). So far, parasporins have been divided into six main classes starting from parasporin 1 (PS1) to PS6, and more than 18 subclasses have been added to the list (Wong *et al.,* 2010; Okassov, 2015). The parasporal proteins of non-cytolitic (non-cyt) and non-insecticidal *B. thuringiensis* strains are highly toxic to human cancer cell lines *in vitro* because of their mammalian cell recognition and killing activity (Kim *et al.,* 2000). Studies in the literature provides an evidence that parasporin activities were observed among the naturally occuring *B. thuringiensis* strains in different countries (Okassov, 2015). Therefore, in this present study, *B. thuringiensis* strains from Bt collection of H. Gunes Molecular Biology Laboratory were characterized based on *cyt* and *parasporin* gene contents as well as *in vitro* cytotoxicity against human cancer cell lines.

## Materials and Methods

***B. thuringiensis* type strains and isolates :** Reference strains *B. thuringiensis* biovar *israelensis* (BGSC 4Q2) and *B. thuringiensis* serovar *dakota* strain 4R2 for parasporin 2 were kindly supplied by Dr. Zeigler (Bacillus Genetic Stock Center, Colombus, OH, USA). Positive controls for *parasporin* 1, 5 and 6 genes were gifted from Dr. Rampersad (University of Texas-Pan American, Edinburg by, TX, USA). Native *B. thuringiensis* strains, used in this study, were originally isolated from boron containing soil samples of Balıkesir and Kütahya Province of Turkey.

**Detection of cyt and parasporin genes :** PCR was performed for detection of *cyt* and *parasporin* genes. Two pairs of primers synthesized from conserved regions of *cyt 1* and *cyt 2* genes were used for detection of *cyt* positive strains (Ibarra *et al.,* 2003). Plasmid DNA was used as DNA template for PCR experiments. PCR reactions were performed in a 50 µl volume containing 200 µM dNTP, 0.5 µM of each primers, 1.5 mM $MgCl_2$ and 2 U of Taq DNA polymerase and 200 ng DNA. Amplification was performed in a thermal cycler (PeqLab, Erlangen, Germany) using a 30 cycles of program. After an initial denaturation step at 95°C for 2 min, each cycle consists of a denaturation step at 95°C for 1 min, a 1 min annealing step (52°C for *cyt* 1 and 50°C for *cyt* 2) and an extention step at 72°C for 1 min and a final extention step at 72°C for 5 min was performed. The primers and amplification conditions for *parasporin* 1, 2, 3 and 4 genes as described by Lenina *et al.* (2014) and for parasporin 5 and 6 genes as described by Ammons *et al.* (2015) were used. After amplification, 10 µl PCR product was electrophoresed on a 1% agarose gel and visualized in a gel documentation system. All reagents for PCR were purchased from Thermo Scientific (Finland).

**Preparation, solubilization and activation of parasporal proteins :** Preparation of activated parasporal proteins was performed with minor modifications following the method of Brasseur *et al.* (2015). Briefly, *B. thuringiensis* isolates were grown at 30°C for 2-4 days on nutrient agar until the cell lysis was completed. Harvested cells were washed two times with sterile distilled water. The spore-crystal pellet was solubilized in 500 µl solubilisation buffer (56 mM $Na_2CO_3$, pH 11, 5 and 11 mM dithiothreitol) at 37°C for one hour. After that, insoluble material was removed by centrifugation at 6000 ×g for 2 min. Supernatant was transferred to a new centrifuge tube and pH was adjusted to 8 with 1 M tris-HCl (pH 4.98). Then, it was digested with proteinase K at a final concentration of 150 µg $ml^{-1}$ for one hour at 37°C. PMSF at 1mM final concentration was added to stop proteolytic reaction. SDS-PAGE analysis were performed to show parasporal protein profiles (Laemmli, 1970). Protein concentration was determined by Bradford method using bovine serum albumin as standard (Bradford, 1976).

**Hemolytic activity assay :** This assay was carried out according to a protocol described by Eren *et al.* (2008). Fresh human red blood cells (RBCs, 30 µl) were suspended in 10 ml of TRIS saline. After washing three times by centrifugation for 5 min at 1500 rpm, it was resuspended in TRIS saline. Solubilized and activated parasporal protein samples (10 µg $ml^{-1}$) were added to 80 µl of the prepared RBC suspension to reach a final volume of 100 µl in a 96 well plate. The plate was incubated at 37°C for 30 min under rotary agitation. After that, the plate was centrifuged (Microcentrifuge 200, Hettich) for 5 min at 3000 rpm and the

supernatants from the wells were transfered to a new plate. In order to determine the hemolysis, absorbance of released hemoglobin was measured at 405 nm using microplate reader (Multiscan FC, Thermo Scientific). A 10 µl Triton-X solution (20%) was added to the above-prepared RBC suspension to achive 100% hemolysis as positive control. TRIS saline without parasporal protein served as negative control. Experiments were performed as triplicate.

**Cells and culture conditions :** Three human cancer cell lines; prostate cancer cell (PC-3), human cervix adenocarcinoma (HeLa) and lung cancer cell (A-549) were used in this study. Normal lung epithelial cells (BEAS-2B) served as control for certain experiments. All the cell lines were originally obtained from ATCC. Cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum, penicillin (100U $ml^{-1}$) and streptomycin (100 µg $ml^{-1}$) in 5% $CO_2$, 95% air in a humified incubator.

**Cytotoxicity assay :** Cytotoxic activity of parasporal protein preparations were determined by MTT assay (Applichem, USA). Each well of 96 well plates (Greiner, Germany) was seeded with 180 µl of cell suspension containing $2\times10^4$ cells and incubated for 24 hr at 37°C in 5% $CO_2$ and 95% air in a humidified incubator. Solubilized and activated *B. thuringiensis* toxins in solubilization buffer were diluted in culture medium and added to the cell culture at a final concentration of 10 µg $ml^{-1}$. After incubating the cells for 24 hr, 10 µl MTT solution (5 mg $ml^{-1}$) was added to each well and incubated for 4 hr at 37 °C. The medium was then discarded and 100 µl DMSO were added to each well in order to dissolve formazan blue crystals. Optical density was determined by reading the absorbance at 540 nm in a microplate reader (Thermo Scientific, Multiscan FC). Each experiment was carried out as triplicate. Cytotoxicity was determined by comparing the optical density of treated cells against the optical density of untreated cells.

**Statistical analysis :** The differences between applications were determined by one way analysis of variance (ANOVA) or Kruskal-Wallis depending on variance was homogenous. Comparision of experimental groups, when using ANOVA, were determined by Dunnett and Tukey tests. Statistical significance level was accepted at *p<0.05, **p<0.01 and ***p<0.001. Data are presented as mean ± SD of three replicates.

## Results and Discussion

Cytocidal activity against human cancer cells was achieved by parasporal proteins from non-incesticidal and non-hemolytic *B. thuringiensis* strains by Okassov *et al.* (2015). Unlike Cry proteins, Cyt proteins exhibit a general cytolytic and hemolytic activity (Guerchicoff *et al.,* 2001; Butko, 2003). Therefore, firstly fourteen native *B. thuringiensis* strains harboring cry 1 and/or cry 2 genes (Şahin *et al.,* 2017) were screened for the presence of *cyt* genes using primers for *cyt 1* and *cyt 2* genes in order to eliminate cytoliytic and hemolytic *B. thuringiensis* strains. In total, five *B. thuringiensis* strains showed amplification with *cyt* gene primers. Bt-BB48 strain harbored only *cyt 1* gene while Bt-KE-6364, Bt-KH 3 and Bt-BB99 strains harbored only *cyt 2* gene and the strain Bt-KH58 contained both *cyt 1* and *cyt 2* genes together (Fig. 2 A). However, 9 strains

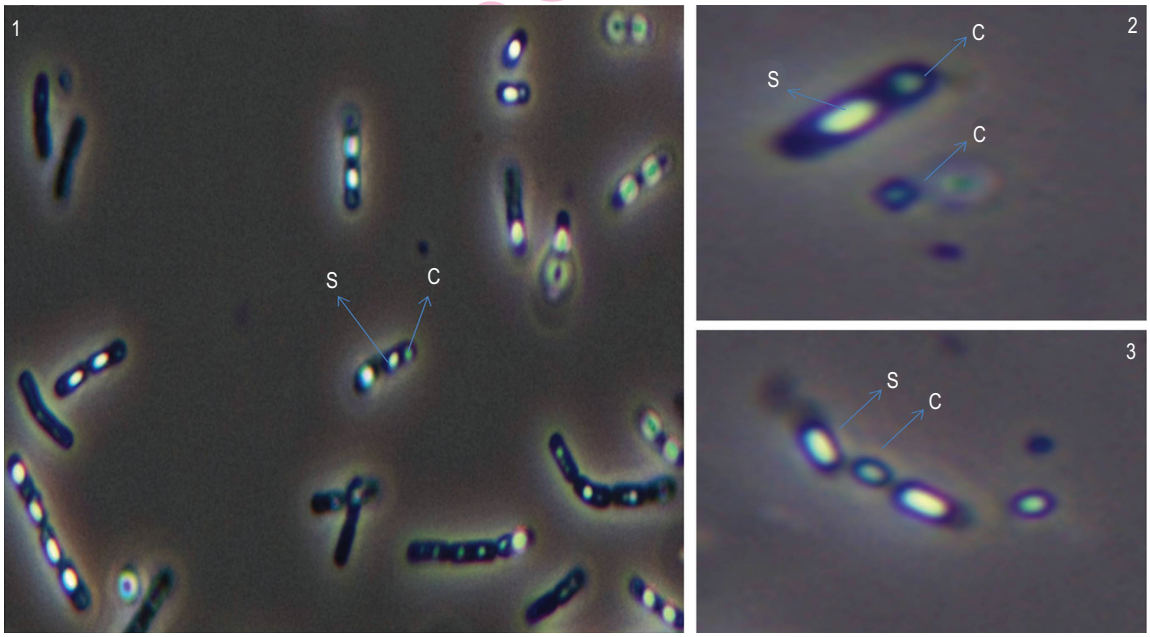



**Fig. 1 :** Phase contrast microscopy photographs of Bt strains: (1) Bt-KE63-64; (2) Bt-KH58 and (3) Bt-Ba14 isolates. S : spore; C : crystal.

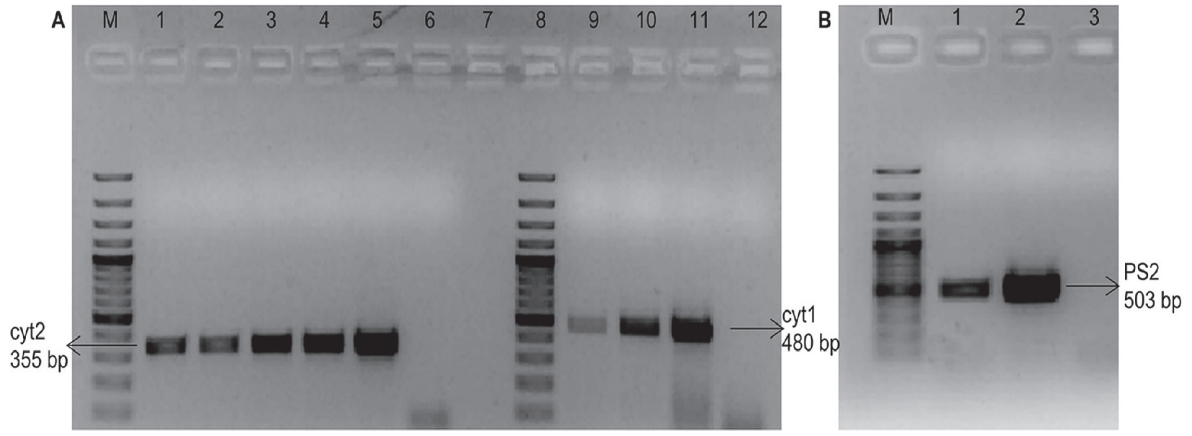



**Fig. 2 :** cyt and *parasporin 2* gene profiles of Bt isolates: (A) Lanes 1-6 show cyt 2 profiles of 1: Bt-KE63-64; 2: Bt-KH58; 3: Bt-KH3; 4: Bt-BB99; 5: Positive control (Bt *israelensis*); 6: Negative control. Lanes 8-12 show *cyt 1* profiles of Bt strains. 8: Marker; 9: Bt-KH 58; 10: Bt-BB4; 11: Positive control (Bt *israelensis*); 12: Negative control. (B) Lanes 1-3 show *parasporin 2* of 1: Bt-Ba14; 2: 4R2 reference strain; 3: Negative control.

showed no amplification for none of the *cyt* genes and therefore; the *cyt* gene negative strains were used for investgation of *in vitro* cytotoxic potential of parasporal proteins. Distrubition of *cyt* genes among *B. thuringiensis* strains change according to the environment. Gao *et al.* (2008) reported that in total of 143 *B. thuringiensis* isolates, only 9 isolates (6%) harbored cyt genes. However, *cyt* gene content of *B. thuringiensis* strains in this study was 35%. Parasporal proteins exhibiting cytocidal activity against leukemia T-cells and other human cancer cells are called parasporins. Based on amino acid identity, there are 6 main

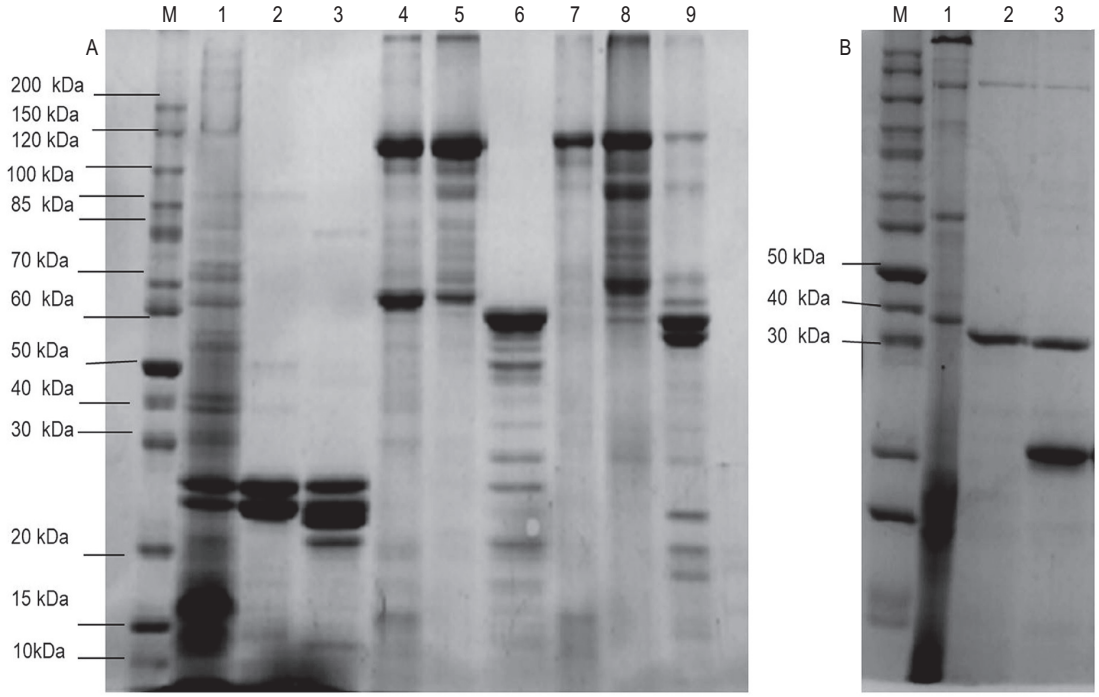



**Fig. 3 :** SDS-PAGE analysis. Protein profiles of Bt strains are given in following order: spore-crystal mixture, alkali solubilized and proteinase K treated parasporal proteins. (A) M: protein molecular weight marker, SM0661 (Fermentas); Lanes 1,2,3: Bt *israelensis;* Lanes 4,5,6: Bt-KH58; Lanes 7,8,9: Bt-KE-6364. (B) M: protein marker; Lanes 1,2,3: Bt-Ba14.

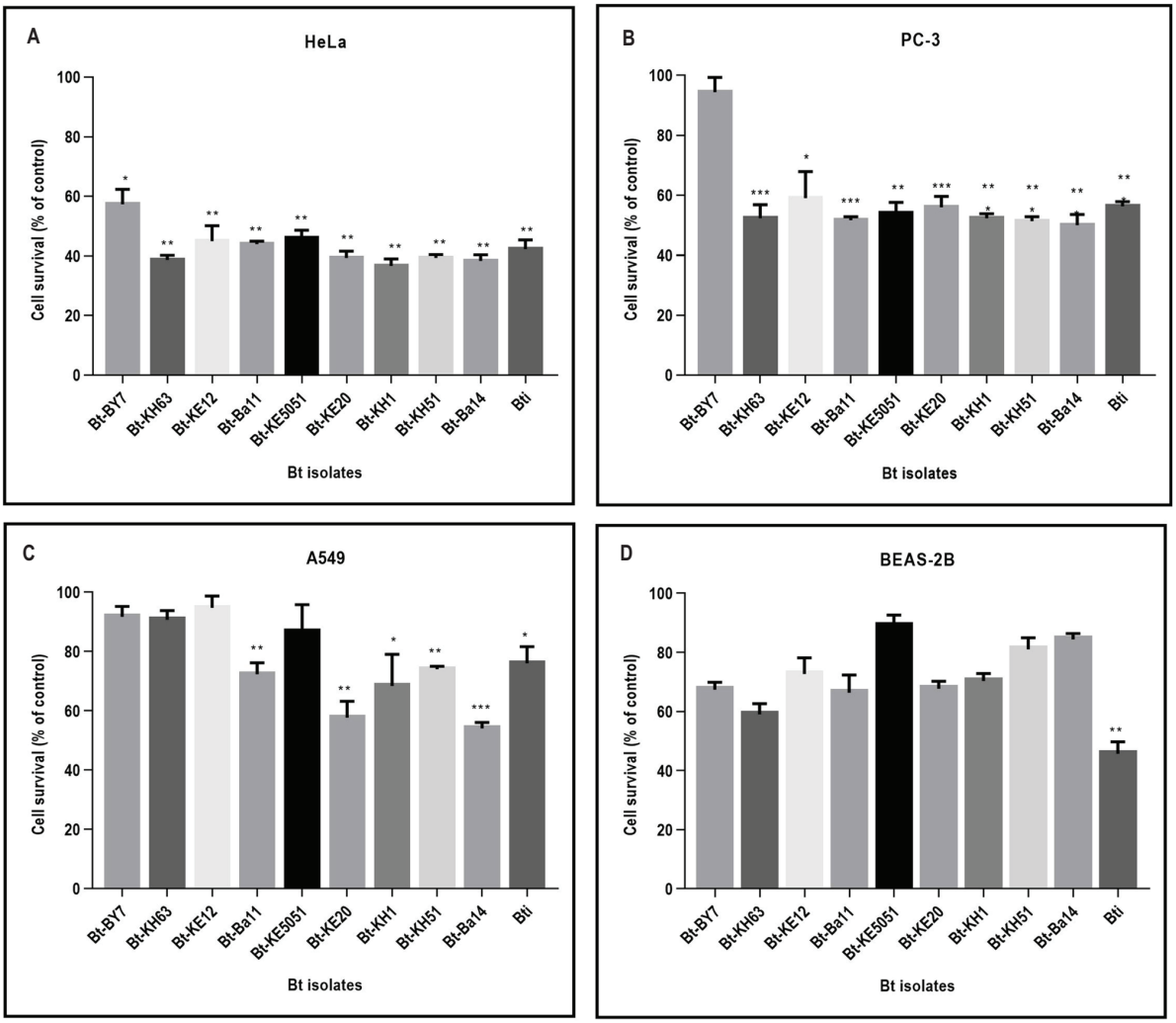



**Fig. 4 :** Cytotoxic effects of parasporal proteins from diferent Bt strains. The cells were plated onto 96-well plates and treated with activated parasporal protein (10 µg ml$^{-1}$) for 24 hr. Cytotoxicity was determined based on MTT assay. Data are means of three replicates±SD. *: P<0.05; **: P<0.01; *** : P<0.001.

classes of parasporins encoded by different parasporin genes. Therefore, PCR analysis of each different *parasporin* genes (from 1 to 6) was carried out to see if these native strains contain any of the known *parasporin* genes (Fig. 2 B). Our findings indicated that Bt-Ba 14 was the only strain harboring *parasporin 2* and exhibiting PCR product at 503 bp, same as the band observed with parasporin 2 positive 4R2 reference strain (Brasseur *et al.,* 2015). On the other hand, rest of the *parasporin* genes were not detected in 14 *B. thuringiensis* strains. Low abundance of parasporin positive *B. thuringiensis* strains in our collection is consistent with the results of Mizuki *et al.* (1999). They showed that among 1744 *B. thuringiensis* strains, only 42 *B. thuringiensis* strains exhibited *in vitro* cytotoxicity against human leukemic T-cells and these strains were *cyt* negative (non-cyt) and non-hemolytic. Similarly, a total of 9 non-cyt Bt strains was identified as non-hemolytic after performing hemolytic activity assay. The spore-crystal mixture was prepared from the sporulated *B. thuringiensis* cultures as described in methods section. Solubilization of parasporal proteins were carried out in different alkaline conditions ranging from pH 9 to pH 12 (data not shown). Because the optimum solubilization was observed at pH 11.5, all solubilization studies were done under this alkaline condition. In addition, the amount of proteinase K for digestion of parasporin was optimized as well. Eventually, solubilized and activated parasporal proteins were prepared from nine *B. thuringiensis* strains.

SDS-PAGE analysis of representative three strains are shown in Fig. 3A. The alkali solubilized protein profiles of isolates revealed that the solubilization process works under the experimental conditions described and removes the unsoluble

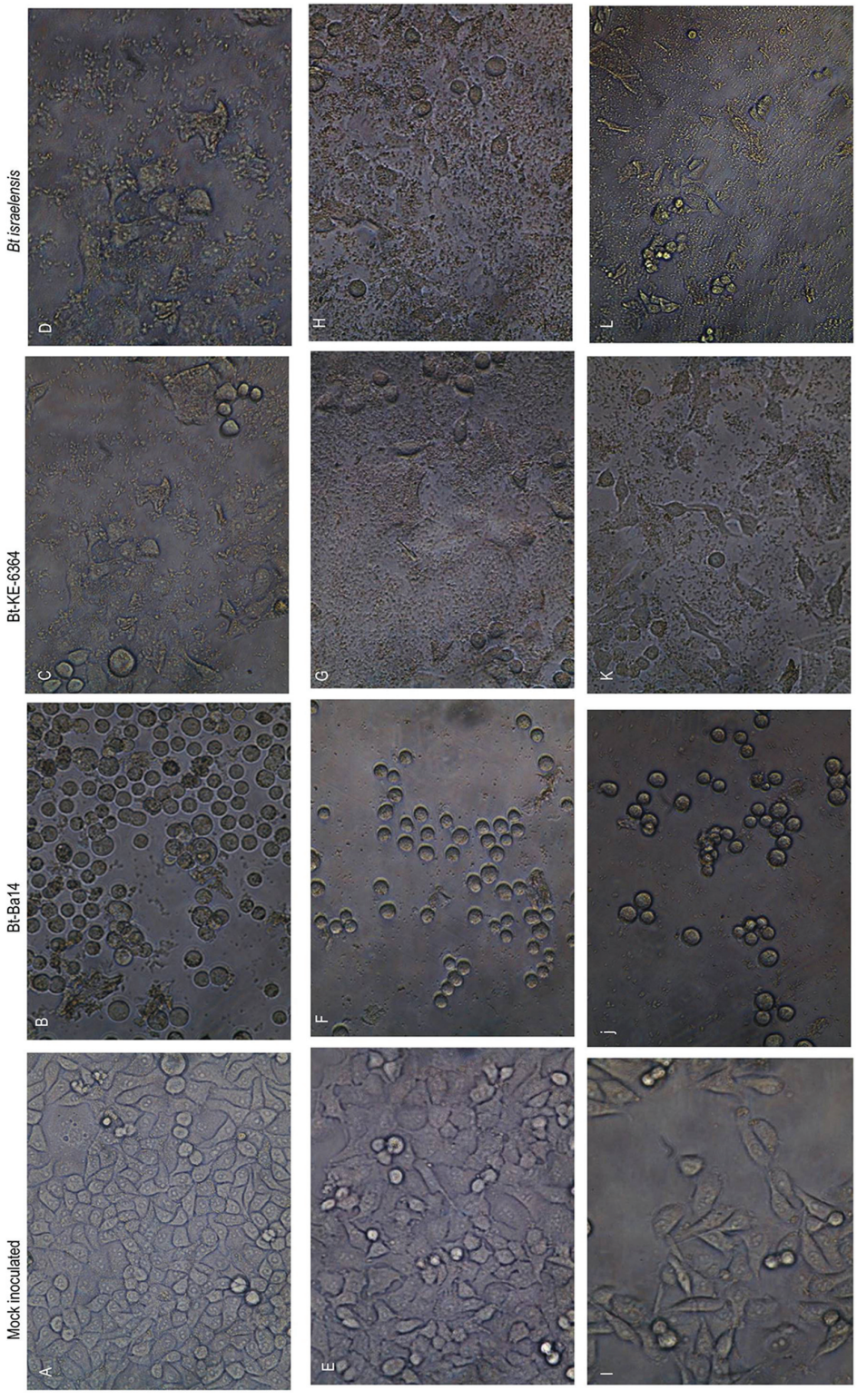



**Fig. 5 :** Morphological changes of cancer cells after exposure to activated parasporal proteins from different Bt strains. Morphopathological effects on HeLa, A549 and PC-3 cells were shown in panelsBF J for Bt-Ba14; CGK for Bt-KE-6364; and DHL for Bt *israelensis*. Panels A, E and I indicates untreated cells. All images were obtained by inverted microscopy at a magnification of 200× after 24 hr incubation of cancer cells in the presence or absence of activated toxin.

proteins. Therefore, characteristic protein bands were fewer than spore crystal mixture of Bt cultures. Fig. 3A shows the protein profile of isolates were different from Bt *israelensis* and from each other.

Moreover, SDS-PAGE analysis of *parasporin 2* positive Bt-Ba14 strain indicates unsolubilised protein band at 37 kDa (Fig. 3B Lane1) as shown before (Brasseur *et al.,* 2015). After solubilization and activation, Parasporin 2 exists as 30 kDa protein (Wong *et al*., 2010). Similarly, Parasporin 2 was obtained at the same molecular weight (Fig. 3 B, Lanes 2, 3).

Before cytotoxicity analysis, parasporal protein preparations from each of the 9 Bt strains negative for *cyt* genes were tested for hemolysis and found that none of them exhibited hemolytic activity (data not shown). Even though only one strain Bt-Ba14 was shown to have one of the known *parasporin* gene, cytotoxic effects of solubilized and activated parasporal proteins from other 8 Bt strains were tested as well because they may contain unidentified cytotoxic protein. Hence, solubilized and activated parasporal proteins from 9 *B. thuringiensis* strains were tested for cytotoxicity against HeLa, PC-3 and A549 cancer cell lines as well as a normal lung epithelial cell line BEAS-2B. Cytotoxic activity of Bt *israelensis* was used as control because of its indiscriminative cytolytic toxicity aganist a broad spectrum of cell lines due to its Cyt 1 and Cyt 2 proteins (Yamashita *et al.,* 2000).

Cell viability rates and the statistical significance of MTT results are shown in Fig. 4. The cytotoxic activities of parasporal proteins were higher on HeLa cells than other cell lines. Parasporal proteins of Bt-KH63, Bt-KH1, Bt-KE20 and Bt-Ba14 strains were highly toxic to HeLa cells with the cell death rates of 61.6, 63.4, 61.4 and 61.6, respectively whereas they were less effective against normal cell line BEAS-2B (Fig. 4A and D). Especially, Bt-Ba14, Bt-KH51, Bt-KE5051 gave rise to less than 18% cytotoxicity against BEAS-2B cells indicating selectivity of these parasporal proteins on cancer cells.

The most cytotoxic parasporal proteins on PC-3 cells were obtained from Bt-Ba14,Bt-Ba11, Bt-KH1, Bt-KH51 and Bt-KH51isolates that showed around 50% cytotoxicity (Fig. 4B). On the other hand, the cytotoxic activity of *B. thuringiensis* strains against A549 cells was lower than other cell lines. Among the parasporal toxins tested, Bt-Ba 14 was most effective and selective toxin against A549 cells with the cell death rate of 48% because it caused 17% cytotoxicity against BEAS-2B cells (Fig. 4D). In short, most of the *B. thuringiensis* strains showed different range of cytotoxicity against the cancer cell lines and especially Bt-Ba14 exhibited a selective cytotoxicity against all 3 cancer cell lines but not against normal cell line BEAS-2B. In other words, cytotoxicity of Bt-Ba14 against all cancer cells was higher than 2.8-fold compared to normal BEAS-2B cell.

Unlike Bt-Ba14, rest of the Bt strains exhibiting cytotoxic activity did not contain any known *parasporin* genes. The reason for this is that they may probably carry parasporal proteins different from currently identified parasporins. In fact, Nadarajah *et al.* (2008) reported no sequence similarity between parasporal protein from Bt-18 and parasporins; however, they showed that polypeptide from Bt-18 share similar properties with parasporin because it is non hemolytic but discriminately cytotoxic against leukemic cell lines. Therefore, further studies focusing on detalied characterization of parasporal proteins especially from Bt-Ba14 may provide a new addition to the parasporin list. Up to date, more than 19 parasporins have been discovered and added to the list of parasporins by the committee (Okumura *et al.,* 2010; Okassov, 2015).

When the morphological changes induced by parasporal proteins from *cyt* positive and *cyt* negative Bt strains were analysed by inverted microscopy, it was observed that cyt positive isolates such as Bt-KE6364 lead to drastic cell lysis (Fig. 5 C,G,K) as it was observed with Bt *israelensis* (Fig. 5 D,H,L). However, parasporal protein from *parasporin 2* positive and *cyt* negative strain Bt-Ba 14 caused cell death with cell balloning (Fig. 5 B,F,J) which is characterized by parasporin proteins (Mizuki *et al.,* 2000; Gao *et al.,* 2008; Ekino *et al.,* 2014).This result indicate that the Bt-Ba 14 strain is a potential candidate for full characterization and production of anti-cancer parasporin against cancer cells.

In conclusion, parasporal proteins from non-cyt and non-hemolytic Bt strains exhibited cytotoxicity against cancer cell lines at different levels. Because Bt-Ba14 strain displayed the most effective and selective cytotoxicity against cancer cell lines compared to a normal cell line, Bt-Ba 14 is a promising candidate for further characterization of mechanism of action on cancer cell death and development of anti-cancer agent.

## Acknowledgment

This work was supported by Scientific Research Projects (BAP) of Mugla Sıtkı Kocman University in Turkey under project number 15/152.

## References

Ammons, D.R., J.D. Short, J. Bailey, G. Hinojosa, L. Tavarez, M. Salazar and J.N. Rampersad: Anti-cancer parasporin toxins are associated with different environments : Discovery of two novel Parasporin 5-like genes. *Curr. Microbiol.,* **72**, 184-189 (2015).

Bradford, M.M.: A rapid sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.,* **72**, 248-254 (1976).

Brasseur, K., P. Auger, E. Asselin, S. Parent, J.C. Côté and M. Sirois: Parasporin-2 from a new *Bacillus thuringiensis* 4R2 strain ınduces caspases activation and apoptosis in human cancer cells. *PLoS One*, **10**, e0135106 (2015).

Butko, P.: Cytolytic toxin Cyt1 A and ıts mechanism of membrane damage: Data and hypotheses. *Appl. Environ. Microbiol.,* **69**, 2415-2422 (2003).

Crickmore, N., D.R. Zeigler, J. Feitelson, E. Schnepf, J. Van Rie, D. Lereclus, J. Baum and D.H. Dean: Revision of nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. *Microbiol. Mol. Biol. Rev.,* **62**, 807-813 (1998).

Ekino, K., S. Okumura, T. Ishikawa, S. Kitada, H. Saitoh, T. Akao, T. Oka, Y. Nomura, M. Ohba, T. Shin and E. Mizuki : Cloning and characterization of a unique cytotoxic protein parasporin-5 produced by *Bacillus thuringiensis* A1100 strain. *Toxins*, **6**, 1882-1895 (2014).

Eren, T., A. Som, J.R. Rennie, C.F. Nelson, Y. Urgina, K. Nusslein, E.B. Coughlin and G.N. Tew : Antibacterial and hemolytic activities of quaternary pyridinium functionalized polynorbornenesa. *Macromol. Chem. Phys.,* **209**, 516–524 (2008).

Gao, M., R. Li, S. Dai, Y. Wu and D. Yi : Diversity of *Bacillus thuringiensis* strains from soil in China and their pesticidal activities. *Biol. Control*, **44**, 380-388 (2008).

Guerchicoff, A., A. Delécluse and C.P. Rubinstein : The *Bacillus thuringiensis cyt* genes for the hemolytic endotoxins constitude a gene family. *Appl. Environ. Microbiol*., **67**, 1090-1096 (2001).

Höfte, H. and H.R. Whiteley : Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiol. Rev.,* **53**, 242–255 (1989).

Ibarra, J., M.C. Rincón, S. Ordúz, D. Noriega, G. Benintende, R. Monnerat and A. Bravo : Diversity of *Bacillus thuringiensis* strains from Latin America with insecticidal activity against different mosquito species. *Appl. Envıron. Mıcrob.,* **69**, 5269-5274 (2003).

Kim, H.S., S. Yamashita, T. Akao, H. Saitoh, K. Higuchi, Y.S. Park, E. Mizuki and M. Ohba : *In vitro* cytotoxicity of non-cyt inclusion proteins of a *Bacillus thuringiensis* isolate against human cells, including cancer cells. *J. Appl. Microbiol*., **89**, 16-23 (2000).

Laemmli, U.K. : Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature,* **227**, 680-685 (1970).

Lenina, N.K., A. Naveenkumar, A.E. Sozhavendan, N. Balakrishnan, V. Balasubramani and V. Udayasuriyan : Characterizatation of parasporin gene harbouring Indian isolates of *Bacillus thuringiensis*. 3 *Biotech.,* **4**, 545-551 (2014).

Mizuki, E., M. Ohba, T. Akao, S. Yamashita, H. Saitoh and Y.S. Park : Unique avtivity associated with non-insecticidal *Bacillus thuringiensis* parasporal inclusions : *In vitro* cell-killing action on human cancer cells. *J. Appl. Microbiol.,* **86**, 477-486 (1999).

Mizuki, E., Y.S. Park, H. Saitoh, S. Yamashita, T. Akao, K. Higuchi and M. Ohba: Parasporin a human leukemic cell-recognizing parasporal protein of *Bacillus thuringiensis*. *Clin. Diagn. Lab. Immunol.,* **7**, 624- 634 (2000).

Nadarajah, V.D., D. Ting, K.K. Chan, S.M. Mohamed, K. Kanakeswary and H.L. Lee : Selective cytotoxic activity aganist leukemic cell lines from mosquitocidal *Bacillus thuringiensis* parasporal inclusions. *Southeast Asian J. Trop. Med. Public Hlth.,* **39**, 235-245 (2008).

Ohba, M., N. Wasano and E. Mizuki : *Bacillus thuringiensis* soil populations naturally occuring in the Ryuksus a subtropic region of Japan. *Microbiol. Res.,* **155**, 17-22 (2000).

Okassov, A.: Parasporins as a new anticancer agents : A review. *J. Buon*., **20**, 5-16 (2015).

Okumura, S., M. Ohba, E. Mizuki, N. Crickmore, J.C. Cote, Y. Nagamatsu, S. Kitada, H. Sakai, K. Harata and T. Shin : Parasporin nomenclature http://parasporin.fitc.pref.fukuoka.jp / Accesed 14 May 2015 (2010).

Palma, L., D. Muñoz, C. Berry, J. Murillo and P. Caballero : *Bacillus thuringiensis* toxins : An overview of their biocidal activity. *Toxins*, **6**, 3296-3325 (2014).

Roh, J.Y., J.Y. Choi, M.S. Li, B.R. Jin and Y.H. Je : *Bacillus thuringiensis* as a specific, safe, and effective tool for insect pest control. *J. Mol. Biol.,* **17**, 547–559 (2007).

Salehi Jouzani, G.H., A. Seifinejad, A. Saeedizadeh, A. Nazarian, M. Yousefloo, S. Soheilivand, M. Mousivand, R. Jahangiri, M. Yazdani, R. Maali Amiri and S. Akbari : Molecular detection of nematicidal crystalliferous *Bacillus thuringiensis* strains of Iran and evaluation of their toxicity on free-living and plant-parasitic nematodes. *Can. J. Microbiol.,* **54**, 812–822 (2008).

Şahin, B., B. Çöl and H. Güneş : *Bacillus thuringiensis* isolation from environments of boron mines and effects of boric acid on bioactivity. *GU J. Sci.,* **30**, 223-234 (2017).

Schnepf, E., N. Crickmore, R.J. Van, D. Lereclus, J. Baum, J. Feitelson, D.R. Zeigler and D.H. Dean : *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiol. Mol. Biol. R.,* **62**, 775-806 (1998).

Soberon, M., J.A. Lopez-Diaz and A. Bravo: Cyt toxins produced by *Bacillus thuringiensis* : A protein fold conserved in several pathogenic microorganisms. *Peptides,* **41**, 87–93 (2012).

Wong, R.S.Y., S.M. Mohamed, V.D. Nadarajah and I.A.T. Tengku : Characterisation of the binding properties of *Bacillus thuringiensis* 18 toxin on leukaemic cells. *J. Exp. Clın. Canc. Res.,* **29**, 86 (2010).

Yamashita, S., T. Akao, E. Mizuki, H. Saitoh, K. Higuchi, Y.S. Park, H.S. Kim and M. Ohba : Characterization of the anti-cancer-cell parasporal proteins of a *Bacillus thuringiensis* isolate. *Can. J. Microbiol.,* **46**, 913–919 (2000).