DETECTION OF *BACILLUS THURINGIENSIS* CRYSTAL PROTEIN CRY51AA1 INTOXICATION *(IN VIVO)* USING ZEBRAFISH MODEL

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ABSTRACT

Bacillus thuringiensis is a ubiquitous a gram-positive, anaerobic, spore-forming bacterium, produces various proteins (toxins) during the stationary and vegetative phase of its growth cycle. Some of these proteins belonging to this group showed weak similarities to ß-barrel pore-forming toxic proteins, such as the cytotoxins of *Pseudomonas aeruginosa*, the epsilon toxin of *Clostridium perfringens*, alpha-toxin of *Clostridium septicum.* In the present study, the intoxication effects of Cry51Aa1 protein produced by *B.thuringiensis* F14-1 strain was evaluated *in vivo*, in order to investigate the ability of activated Cry51Aa1 to make a change in size or functions of zebrafish cardiovascular system and induces toxicity in adult zebrafish. Using *in vivo* imaging we observed that Cry51Aa1 has no scientific effect on cardiac function and development of zebra fish embryo or have toxic effect on adult in spite of use high concentration, so it is safe to use and a potentially effective agent in breast cancer therapy.

INTRODUCTION

Bacillus thuringiensis is an ubiquitous a gram-positive, anaerobic, spore-forming bacterium belonging to the *Bacillus cereus* group, produces various toxins during the stationary and vegetative phase of its growth cycle and was first isolated in Japan from diseased larvae of the silkworm, Bombyx Mori, as an entomopathogenic bacterium [1]. Under aerobic conditions, it produces a spore along with one or several parasporal inclusion crystals proteins during the sporulation phase. This phase consists of seven

stages, Parasporal protein synthesis start at about stage II or III of its phase[2, 3]. Parasporal protein is δ-endotoxins classified into two families Cry and Cyt proteins [4] Cry protein is the predominant type and some of this proteins belonging to this group showed similarities to the Mosquitocidal Mtx2 and Mtx3 proteins from *Bacillus sphaericus*, and more members of this group have weak similarities to ß-barrel pore-forming toxic proteins, such as the cytotoxins of *Pseudomonas aeruginosa*, the epsilon toxin of *Clostridium perfringens*, alpha-toxin of *Clostridium septicum*, and aerolysin of *Aeromonas hydrophila* [5]. The natural environments *B. thuringiensis* isolates with no insecticidal Cry proteins outnumber the insecticidal ones [6, 7], led to a raised question about the possible biological role of these bacteria. Although the studies have started since the beginning of the last third of the last century in various parts of the world to know their effectiveness against cancer, but much of the work being done in Japan since 1990 which their research culminated by [8] invention, when they found, a kind of non-pathogenic, non-insecticidal *B. Thuringiensis* parasporal protein characterized by their ability to recognize and kill cancer cell when digested by proteases in alkaline medium, called later parasporin [9]. Parasporin was a novel biological activity of Cry proteins undiscovered before [10, 11], [12], and heterogeneous in their cytotoxicity, some kill limited kind, whereas others kill many types of cancer cell line [13], which suggested that different modes of action might existed against a certain target organism, or similar mechanism of toxicity might worked against totally distinct species due to the mode of toxin-receptor binding.

Bacillus. thuringiensis strain F14-1 were isolated and screened from a large number of strains from soil samples in northern China, produces Cry51Aa1 has no obvious insecticidal and hemolytic activity, but exhibits cytotoxicity towards several types of cancer cells, especially breast cancer cell MDAMB-231 and induced cell apoptosis[14, 15] . Identification of systemic targets of bacterial toxins has been limited, because it is difficult to directly visualize the impact of these proteins on major organs in real time. To overcome this problem, zebrafish embryos were used to characterize the systemic impact of intoxication in real time[16]. Unlike other vertebrates, zebrafish embryos are transparent, and major organs can be visualized by standard light microscopy[17]. Thus, zebrafish embryos provide a unique system for directly visualizing the temporal and

spatial effects of different toxins. By using the zebrafish embryo as a model, in the current work, we have found that Cry51Aa1 has no functions as potent cardiotoxin, or reducing blood flow and ventricle contraction. These findings provide important insight into the *in vivo* activities of Cry51Aa1 using the zebrafish embryo.

MATERIAL AND METHODS

Bacillus thuringiensis culture, activation and purification

Bacillus thuringiensis strain F14-1 that used in this study was obtained from the Agricultural University Wuhan, Hubei, China. It was grown at 28- 30 °C on nutrient agar (pH 7.6). Crystal/spore preparations from *Bacillus thuringiensis* strains and positive colonies were prepared as previously described [18]. Progress of sporulation was monitored by microscopy. When 80 to 90% of the cells had reached sporulation, cultures were harvested and further processed. The amount of crystal protein was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by densitometry, using a bovine serum albumin calibration curve.

Protease treatments.

The crystal protein expressed in BMB171 was partially purified and solubilized with carbonate buffer (pH 10.0) at 37 °C for 1 h. Solubilized proteins were treated at pH 8.0 with trypsin (final conc.: 0.025, 0.25, 2.5 and 25 mg/ml) at 37°C for 90 min, and then phenylmethylsulfonyl fluoride (PMSF, final conc.: 1 mM/ml) was added to the solution at to stop the proteolytic reaction as described previously[14].

Labeling proteins with NHS – Rhodamine.

Protein was labeled with NHS- Rhodamine according to manufacturer's instruction, briefly, Cry51Aa1 was dissolved in 20 mM HEPES and mixed well in reaction tube with NHS-Rhodamine which reconstituted with 100µl of DMSO, and incubated at room temperature for 2h. Then, non-reacted NHS-Rhodamine was removed

by dialysis and protein concentration was calculated by BCA protein assay reagent kit (product No.23225) protein was stored at 4ºC protected from light until uses

Zebrafish Maintenance and Care.

Wild-type Zebrafish used as object in this study was purchased from the Institute of hydrobiology, Chinese academy of Science, Wuhan, P. R. China. Zebrafish were maintained at 28.5°C on a 14-h light_10-h dark cycle, embryo collection, and preparation was performed as described[19].

Treatment of Zebrafish embryos with Cry51Aa1 protein.

Zebra fish embryos were placed into a 96-well plate (five embryos per well) 24 h after fertilization and allowed to incubate with labeled cry51A1 (1, 10, 20 and 100µg/ml) for 24 h, 48, and 72h .No treated zebra fish were used as Control. Subsequently, zebra fish were rinsed 10 times in embryo water for 20 min and visualized by using an Olympus (Melville, NY) BX81 ep- fluorescent microscope.

Treatment of adult Zebrafish with Cry51Aa1 protein.

Fish samples were divided into five groups, each containing 50 fishes in an aquarium of 20-liter capacity. Group I was held in tap water as control and other groups were exposed to Cry51Aa1 concentration 1, 10, 20 and 100µg/ml for 24, 48, and 72h. At the end of each exposure period, some of fish randomly were removed from each tank to dissect and other was left to growth. Each experiment was repeated three times.

RESULT

Activated rhodamine Cry51Aa1 localization in zebrafish Embryos.

To determine the localization of Cry51Aa1, zebrafish were treated with different concentration (1, 10, 20, 100 µg/ml) Cry51Aa1 and examined by fluorescence microscopy for sites of toxin tropism. The toxin primarily localizing to the yolk-sac, pericardial, and cardiac regions of the zebrafish (data not show).

Morphological analysis of activated Cry51Aa1 on zebrafish cardiovascular system.

For the initial characterization of the cytotoxicity induced by the Cry51Aa1 in zebrafish for cardiac damage, embryos were collected 24 h after fertilization and exposed to different concentration (1, 10, 20, 100 µg/ml) of protein under a phase contrast microscope. After 72 h treatment, as shown in Fig.1, in control and treated zebrafish embryos, there was no dynamic change in size or decrease in ventricle chamber contractility, indicating that no intoxication effects of Cry51Aa1 on particular stage of development. Collectively, these observed indicated Cry51Aa1 has no effect on cardiac function and development.

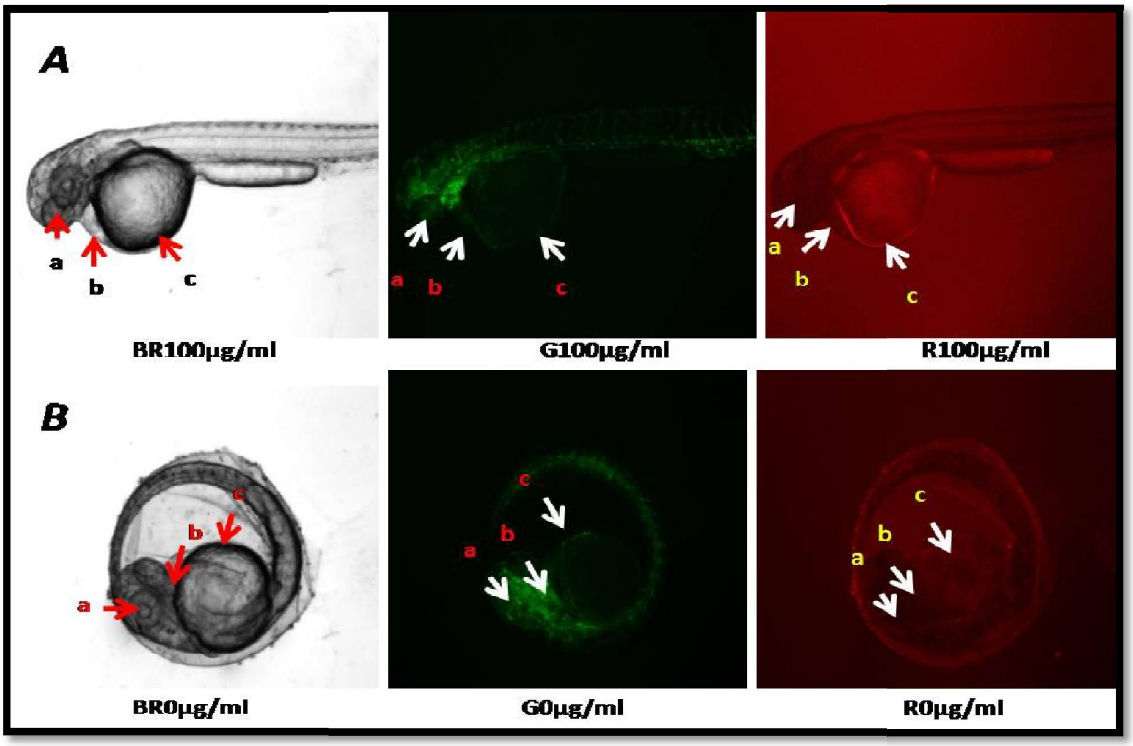

Figure1. Fluorescence images of the morphological analysis of rhodamine-labeled activated Cry51Aa1 in zebrafish. Activated Cry51Aa1 was labeled by rhodamine and treated the zebrafish for 24, 48, and 72h. **(*A*)** Zebrafish embryos treated with 100µL labeled Cry51Aa1 for 72 h. **(*B*)** Untreated (control) Zebrafish for 72h. Arrows a, b, and c denote the eye heart, and yolksac of zebrafish, respectively. (BR) Brightfield image of zebrafish.

Cytotoxic effect of Cry51Aa1 on adult Zebrafish.

We examined the *in vivo* cytotoxitic of Cry51Aa1 to zebrafish embryos and found that Cry51Aa1 had no obvious toxicity to zebrafish embryos in the presence of Cry51Aa1 protein and the results showed that zebrafish embryos survival was not significantly affected by Cry51Aa1 treatment, even in the highest concentration for 72h, as showed in Figure2. On the other hand, all the fish that were dissected was microscopically visualized and showed no signs of poisoning.

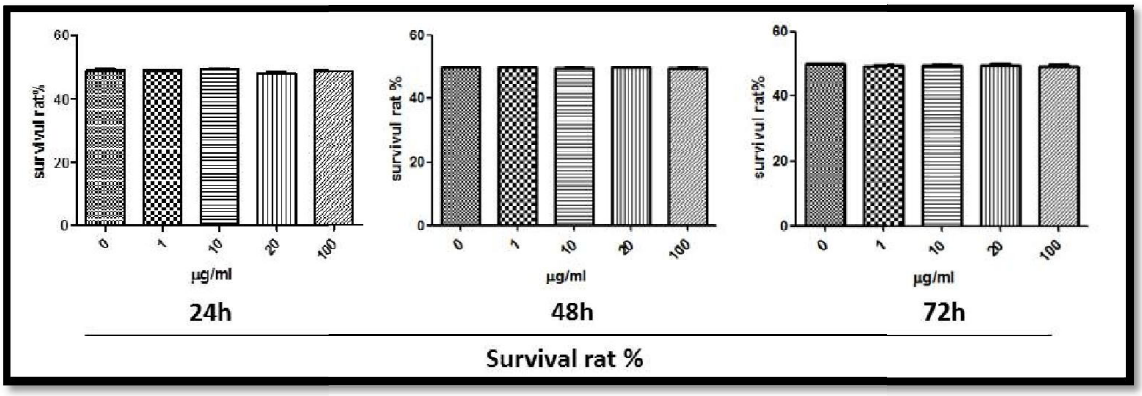

Figure2. Cytotoxitic analysis of activated Cry51Aa1 against adult Zebrafish.

DISCUSSION

Our previous[14] work convey us a concept that Cry51Aa1 is possibly a new Parasporin. Furthermore, marked differences are evident in the anti-cancer cytotoxicity spectra between Cry51Aa1 and other parasporins and MDA-MB-231 is extremely sensitive to Cry51Aa1 since the treatment of activated Cry51Aa1 low to 1 µg/ml concentration could exhibit activity against MDA-MB-231 but other cell types could not. Cry51Aa1 is a novel parasporal protein which can inhibit cancer cell survival, induce apoptosis, which may be a potentially effective agent in cancer therapy [14, 15]. Bacterial toxins are part of many infections and are very important for colonization, immune evasion, and disease progression. Thus, toxin immunogenicity and action mechanisms have been studied for long time, resulting in new vaccines and an understanding of these virulence factors at the molecular level. However, despite many advances in the study of bacterial toxins, little is known about types of targeted cell in vivo [20]. In contrast with, the embryo of zebrafish gives several featured advantages over these traditional models.

Furthermore, having many of the major organs found in humans, zebrafish embryos are diaphanous, which allows direct visualization of labeled toxin and toxin-induced changes in anatomy and physiology[19]. In fact, these same traits have made the zebrafish a widely passable model for the investigations of embryonic development and genetics [21], infectious diseases[22], and the systemic effect of intoxication in real time[16] . A previous study revered that *Clostridium difficile* toxin B (TcdB) was found to localize at the pericardial region, and zebrafish exhibited the first signs of cardiovascular damage, including a 90% reduction in systemic blood flow and a 20% reduction in heart rate. Within 72 h of exposure to TcdB, the ventricle chamber of the heart became deformed and was unable to contract or pump blood, and the fish exhibited extensive pericardial edema[16]. In our observations of the effect of Cry51Aa1 treatment on zebrafish embryos, we found that no scientific effect on cardiac function and development as show in fig.1 because it has no cardiovascular damage or any change in cardiac shape or size[16]. On the other hand, results showed that zebrafish embryos survival was not significantly affected by Cry51Aa1 treatment, even in the highest concentration. Taken together, these findings suggested that Cry51Aa1 may be a significant therapeutic activity[14, 15]

الكشف عن سمية بروتين Cry51Aa1 المستخلص من جرثومة *Bacillus thuringiensis* F-14 داخل الجسم) بأستخدام نموذج اسماك الزيبرا (الزرد)

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الخلاصة

عصيات ثورينجينزيس هي جراثيم واسعة الانتشار موجبة لصبغة جرام لاهوائية،لها القابلية لتشكيل الابواغ، وتنتج انواع مختلفة من البروتينات (السموم) خلال المرحلة الثابتة والنباتية من دورة نموها. بعض من هذه البروتينات تظهر صفات ضعيفة الشبه مع بروتينات بيتا المسامية السامة للبرميات مثل السموم الخلوية لجراثيم السيودوموناس أيروجينوسا، سموم الإبسيلون التي تنتجها جراثيم الكلوستريديوم بيرفرينجنس، وكذلك سموم الألفا المنتجة من قبل جراثيم الكلوستريديوم سيبتيكوم.

دراستنا الحالية تتركز لتقييم التأثير السمي لبروتين *Bacillus thuringiensis* F-14 Cry51Aa1

من خلال التحقق داخل الجسم من قابلية هذا السم على احداث تغيرفي حجم او وظيفة جهاز القلب والاوعية الدموية لاسماك الزيبرا (الزرد) باستخدام طريقة التصوير الداخلي بواسطة المكروسكوب النقيض او احداث تسمم لها عند تغذية الاسماك البالغة بهذا السم.

لقد اظهرت النتائج ان لاوجود لاي تأثير سمي لهذا السم على وظيفة وتطورالقلب في اجنة اسماك الزيبرا او على الاسماك البالغة على الرغم من استخدامه بجرعات عالية مما يجعله آمن للاستخدام وعامل يحتمل أن يكون فعالا في علاج سرطان الثدي.

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