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Full Length Research Paper

Phenotypic characterization and phylogenetic analysis of a virulent *Bacillus cereus* strain from the Tiger frog, *Hoplobatrachus rugulosus* Wiegmann

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Bacillus cereus **is a common opportunistic human pathogen well known as a food contaminant. In recent years, tiger frog farming has been undertaken in China and many other countries to meet human food habits. However, there is no definitive data to indicate the contamination of potentially pathogenic *B. cereus* strains to fresh cultured tiger frogs. In this study, a virulent *B. cereus* strain W10 with hemolytic activity was first isolated from the liver of cultured tiger frogs. It was initially identified based on the spore-forming characterization and phenotypic characterization using API identification kits and its taxonomic position, was further determined by nucleotide blast search in NCBI website and phylogenetic analysis. The constructed phylogenetic tree using neighbor-joining method further showed that strain W10 was closely related to *B. cereus* strain F46 (GenBank accession no. EF203906), which was isolated from the contaminated food. To further confirm the virulence and potential danger of strain W10, its LD_{50} value was tested to be 5.22×10^4 cfu/g, which indicated that strain W10 had strong potential virulence to healthy tiger frogs. Pathological changes were observed in liver and body surface of the challenged tiger frogs. Thus, as a potential pathogen of a zoonotic disease, the distribution of the virulent *B. cereus* strain, may be a threat to the safety of tiger frog food and more importance should be attached to the virulent *B. cereus* strain in tiger frog farming.**

Key words: Tiger frog, *Bacillus cereus*, phenotypic characterization, phylogenetic analysis.

INTRODUCTION

The tiger frog (*Hoplobatrachus rugulosus* Wiegmann) is widely distributed in the southern part of China, Myanmar, Thailand, Vietnam and Malaysia (Shao et al., 2009). However, due to the habitat fragment and destruction, over-hunting by humans, the wild tiger frog population decreased 30% in the last three decades and consequently, it has been listed in Appendix II of Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) and as one of the Class II national protected species in China (Shao et al., 2009). Thus, tiger frog farming has been undertaken in China and many other countries, so as to protect resources of the endangered species and meet human food habits.

The recent economic growth in China and many other countries has improved the standard of living and increased demands for cultured tiger frog products in the national market, owing to the traditional food culture. Tiger frog farming has developed rapidly since the 1990s and is becoming a vigorous industry (Ling et al., 2008). However, the rapid expansion and intensification of tiger frog farming, has led to a series of problems including the

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occurrence of water pollution, various diseases and drug abuse etc., which pollute the tiger frog's total quality. Thus, the safety of cultured tiger frog products has to be paid more attention for the sustainable development of tiger frog industry all over the world.

Bacillus cereus is a common opportunistic human pathogen, well known as a food contaminant. *B. cereus* has been reported as the causative agent in 1 to 22% of food-borne outbreaks in Europe, Japan and North America over the period 1960 to 1992, and in Norway and the Netherlands, it is the most frequently isolated bacterial food-borne pathogen (Pirhonen et al., 2005). Food-borne diseases caused by *B. cereus* are notoriously classified as emetic and diarrheal syndromes. The emetic syndrome is due to cereulide, a heat-stable emetic toxin which causes vomiting. The diarrheal syndrome is apparently owing to several enterotoxins such as hemolysin BL, enterotoxin T, enterotoxin FM, cytotoxin K and the non-hemolytic enterotoxin, which are all involved in *B. cereus* food poisoning (Ghelardi et al., 2002).

However, there is no definitive data to indicate the contamination of potentially pathogenic *B. cereus* strains to fresh cultured tiger frogs. In this paper, a virulent *B. cereus* strain W10 was isolated from the liver of cultured tiger frogs in Qingpu District, Shanghai China, its phenotypic characterization was studied using API identification kits and the phylogenetic analysis was also conducted, to determine its taxonomic position on its partial 16S rDNA sequence. As far as we know, this is the first report on a virulent *B. cereus* strain from the cultured tiger frog. Besides, as a potential pathogen of a zoonotic disease, more importance should be attached to the virulent *B. cereus* strain, for its threat to the safety of tiger frog food and human health.

MATERIALS AND METHODS

Tiger frog samples

10 living tiger frogs (100±10 g in weight) were obtained as samples from concrete ponds of Qingpu Modern Agricultural Development Co., LTD. in Shanghai China during August 2009, where 54,000 tiger frogs were cultured.

Isolation of bacteria

Five sampled tiger frogs were first disinfected with 75% alcohol and dissected in the laboratory. The internal organs including livers were cut and heated for 10 min at 80℃ to destroy vegetative bacteria and fungi to make easier isolation of bacilli with spores that survive the heat pretreatment. The heat-treated internal organ samples were then incubated on rabbit blood agar (RBA) plates (Sinopharm Chemical Reagent Co., Ltd) at 28℃ for 48-72 h, and the predominant uniform colonies were removed for further culture. The pure cultures were obtained by repeated re-isolation and incubation on RBA plates at 28℃ for 48 h. A strain of Bacillus with hemolytic activity was isolated from the heat-treated liver samples using the heat treatment method of selection and enrichment.

Morphological observation

The Bacillus isolate was inoculated and cultured on RBA plates at 28℃ for 24 h, then endospore-stained and observed under light microscope as described by Shen et al. (1999).

Phenotypic characterization and identification using API identification kits

The Bacillus isolate was grown on nutrition agar (NA) plates (Sinopharm Chemical Reagent Co., Ltd) at 28℃ for 24 h, and then the bacterial suspension was used to inoculate the 50 CHB/E API strip (Bio-Merieux, SA) following the manufacturer's instruction. The strip was incubated at 28℃ and observed after 48 h for checking against the API identification index and database.

DNA extract, PCR and sequencing

The Genomic DNA was extracted from pure culture of the Bacillus isolate using genomic DNA extraction kit following instructions of the manufacturer (Shanghai Sangon Biological Engineering Technology and Services Co., Ltd.). The 1.5 kb 16S rDNA gene were amplified by PCR using a pair of universal bacterial 16S rDNA gene primers (27f): 5'-AGAGTTTGATCCTGGCTCAG-3' and (1492r) : 5'-TACGGCTACCTTGTTACGACTT-3'. The PCR was carried out according to Nduhiu et al. (2009). Briefly, 1 µl of the DNA extract was amplified in a 25 µl reaction mix containing 16.75 µl sterilized distilled water, 2.5 µl deoxyribonucleoside triphosphate (dNTP 10 mM), 2.5 µl 10 × buffer, 1 µl $MgCl_2$ (50 mM), 0.5 µl of each primer (10 mM), 0.25 µl (1 U) ExTaq DNA polymerase. Amplification was done using 35 cycles of denaturation at 95℃ for 1 min, annealing at 60℃ for 1 min and extension at 72℃ for 1.5 min followed by a final extension 72℃ for 7 min, using a PCR minicycler (Eppendorf Ltd., Germany). The PCR product was electrophoresed on 1% agarose gel and visualized via ultraviolet trans-illumination. Sequencing was performed by a fluorescent labeled dideoxynucleotides termination method (with BigDye terminator) on ABI 3730 automated DNA Sequencer.

Phylogenetic analysis

The partial 16S rDNA sequence was assembled using MegAlign, Editseq and Seqman software with a power Macintosh computer. Searches were done against the National Centre for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST) program. The phylogenetic tree from partial 16S rDNA sequence of the Bacillus isolate's product and the homologous sequences were further constructed using neighbor-joining method.

Assay for bacterial virulence

32 healthy tiger frogs (100 ± 10 g in weight) were obtained from Qingpu Modern Agricultural Development Co., LTD. in Shanghai China, and were respectively maintained in 4 aquaria (10 tiger frogs per aquaria) supplied with 100 L de-chlorinated tap water at 25 to 28℃. 8 healthy tiger frogs were respectively injected intra-muscularly with 0.3 ml of the Bacillus isolate's live cells (the final concentration was 5.0×107 cfu/ml). Another 8 healthy tiger frogs were injected with 0.3 ml of sterile saline as the control. The experimental tiger frogs were observed daily for 7 days. Dead tiger frogs were immediately removed for pathogen isolation according to Bucke (1989) and the signs and mortalities were recorded.

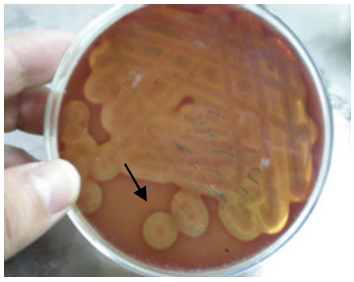

Figure 1. Hemolytic rings of the virulent strain W10 grown on RBA plates.

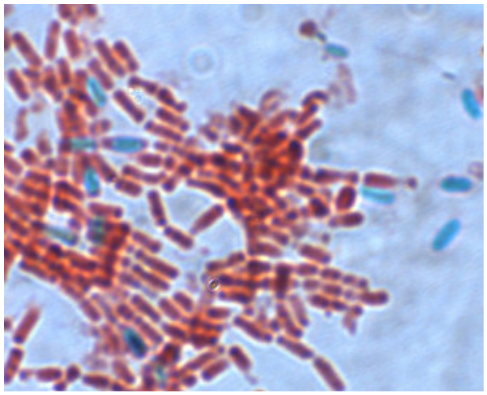

Figure 2. Endospore-stained micrographs of the virulent strain W10: Arrow indicated the endospore (×1000).

Table 1. Phenotypic characterization of strain W10 in comparison with the type strain ATCC14579 of *B. cereus*.

Test item	Reaction	
	W10	ATCC14579
Control	-	-
Glycerin	+	+
Erythritol	-	-
D-arabinose	-	-
L-arabinose	-	-
D-ribose	+	+
D-xlyose	±	±
L-xylose	-	-
D-adonitol	-	-
Methyl-βD-xylo-pyranosid	-	-
D-galactose	±	±
D-glukose	+	+
D-fructose	+	+
D-mannose	±	±
L-sorbose	-	-
L-rhamnose	-	-
Dulcitol	-	-
Inositol	-	-
D-mannitol	-	-
D-sorbitol	-	-
Methyl-αD-mannopyranosid	-	-
Methyl-αD-glucopyranosid	-	-
N-acetylglucosamin	+	+
Amygdalin	-	+
Arbutin	+	+
Esculin	+	+
Salicin	+	+
D-cellobiose	+	+
D-maltose	+	+
D-lactose	+	+
D-melibiose	+	+
D-saccharose	+	+
D-trehalose	+	+
Inulin	-	-
D-melezitose	-	-
D-raffinose	-	-
Amidon	-	+
Glycogen	-	+
Xylit	-	-
Gentiobiose	-	-
D-turanose	+	+
D-lyxose	-	-
D-tagatose	-	-
D-fucose	-	-
L-fucose	+	+
D-arabitol	-	-
L-arabitol	-	-
Kaliumgluconat	+	+
Kalium-2-ketogluconat	-	-
Kalium-5-ketogluconat	-	-
Catalase	+	+

Table 1. Contd.

+: positive reaction; ±: weak positive reaction; -: negative reaction.

After the virulence assay, another 80 healthy tiger frogs were sampled and the test for the mean lethal dose (LD_{50}) value of the Bacillus isolate was further conducted by injection challenge as described above (the final concentration was 5.0×10^5~5.0×10^9 cfu/ml), and the LD_{50} value was calculated using the Probit method described by Won and Park (2008). Each experiment was conducted twice.

RESULTS

Characterization and identification of the isolate

Bacillus isolate W10 could form gray, opaque colonies on RBA plates and produce a typical hemolytic ring (Figure 1). Under light microscope, isolate W10 was a rod-shaped bacterium with the central spore (Figure 2). The API identification kits identified isolate W10 as *B. cereus* (Table 1) and it showed an identity of 94% with the type

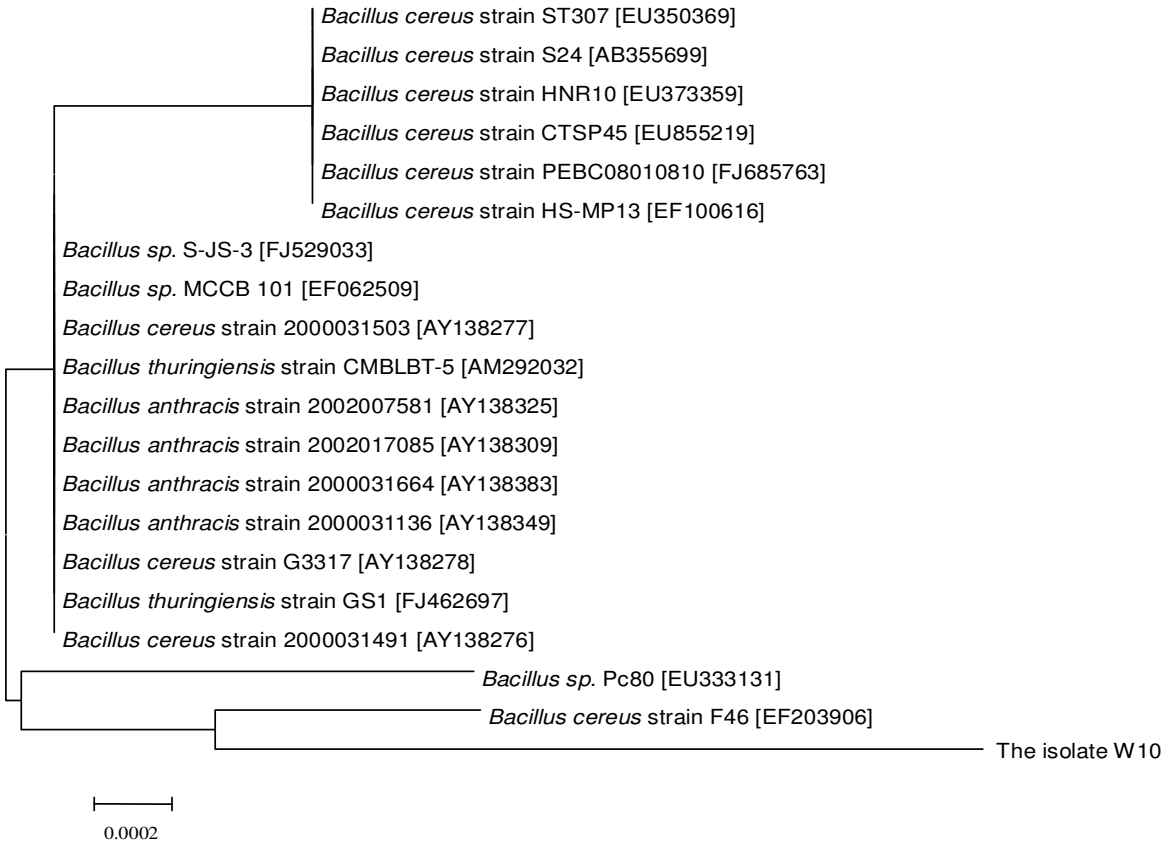

Figure 3. The Phylogenetic tree constructed using neighbor-joining method.

strain ATCC14579 in phenotypic characterization.

Isolate W10 and the type strain ATCC14579 were found both positive for catalase, glycerin, D-ribose, D-xlyose, D-galactose, D-glukose, D-frucotse, D-mannose, N-acetylglucosamin, arbutin, esculin, salicin, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-saccharose, D-trehalose, D-turanose, L-fucose, kaliumgluconat and negative for erythritol, D-arabinose, L-arabinose, L-xylose, D-adonitol, Methyl-βD-xylopyranosid, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, Methyl-αD-mannopyranosid, Methyl-αD-glucopyranosid, inulin, D-melezitose, D-raffinose, xylit, gentiobiose, D-lyxose, D-tagatose, D-fucose, D-arabitol, L-arabitol, kalium-2-ketogluconat and Kalium-5-ketogluconat. However, there were some differences between isolate W10 and the type strain ATCC14579. For example, in contrast to the type strain ATCC14579, isolate W10 was unable to ferment glycogen.

Phylogenetic analysis

The 1.5 kb 16S rDNA sequence of isolate W10 was submitted to GenBank database with the accession no. GU332642. Similarities between the 16S rDNA sequence of isolate W10 and those of *B. cereus* strains in the GenBank database were 99.0%, which proved the initial identification. The constructed phylogenetic tree using neighbor-joining method further showed that isolate W10 was closely related to *B. cereus* strain F46 (GenBank accession no. EF203906) (Figure 3), which was isolated from the contaminated food. The identification result from phylogenetic analysis was consistent with that found through API identification kits.

Virulence of the isolate

After the injection challenge, 68.75% of the test, healthy tiger frogs acutely died (Table 2), which demonstrated that isolate W10 was virulent for the tiger frog at certain concentration of live cells. The challenged tiger frog typically showed the signs of apparent dull black liver and hemorrhage on the head and mouth corner (Figure 4). No acute mortality or visible changes were observed in the control tiger frogs. In addition, the LD_{50} value of isolate W10 was further tested to be 1.74×10^{7} cfu/ml (5.22×10^{4} cfu/g) according to the correlation curve and regression equation of the live cell concentration and mortality (Figure 5) which indicated that isolate W10 was a highly virulent strain according to Mittal et al. (1980), and had

Table 2. Virulence of strain W10 to tiger frogs.

Group	Concentration (cfu/ml)	Frog no.	Death per day							Average mortality (%)
			1	2	3	4	5	6	7	
Bio-saline	0	8	0	0	0	0	0	0	0	0
		8	0	0	0	0	0	0	0	
W10	5.0×10^{7}	8	0	2	2	1	1	0	0	68.75
		8	1	2	1	1	0	0	0	

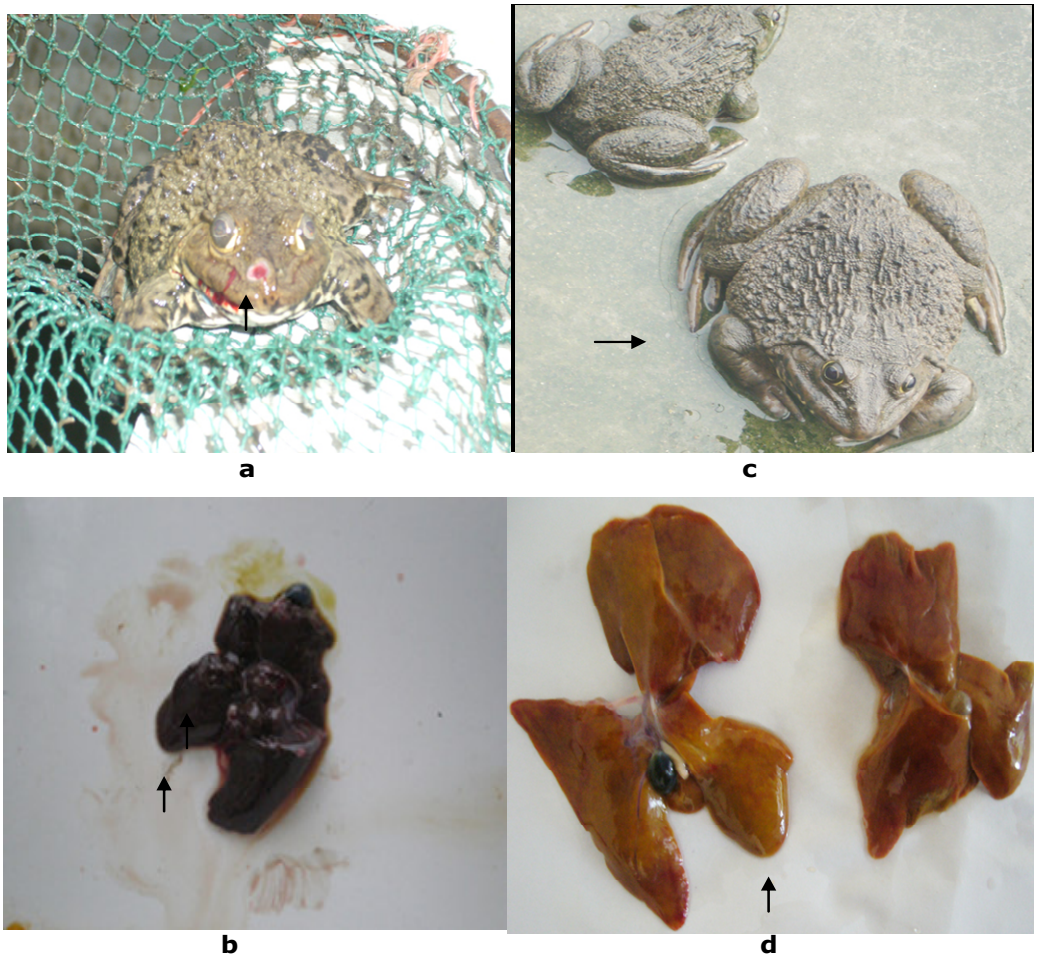

Figure 4. The typical symptoms of the challenged tiger frogs, which indicated the typical signs of external hemorrhages located on the head and mouth corner (a) and the dull black liver (b). The healthy tiger frog (c) and its liver (d) were shown as control.

strong potential pathogenicity to the healthy tiger frogs.

DISCUSSION

The cultured tiger frogs are popular aquatic food in the world market due to the traditional food culture. Thus, more attention should be paid to their safety. So far, some virulent bacteria such as *Elizabethkingia meningoseptica* and *Laribacter hongkongensis* were reported to have contaminated the cultured tiger frog products (Xie et al., 2009; Lau et al., 2009). However, no relevant information is available about the contaminated tiger frog food by virulent *B. cereus* strains. In this study, we isolated a virulent strain of *B. cereus* from the cultured tiger frogs in China. This is the first report on a virulent *B. cereus* strain from the cultured tiger frogs.

B. cereus is widely distributed in the natural

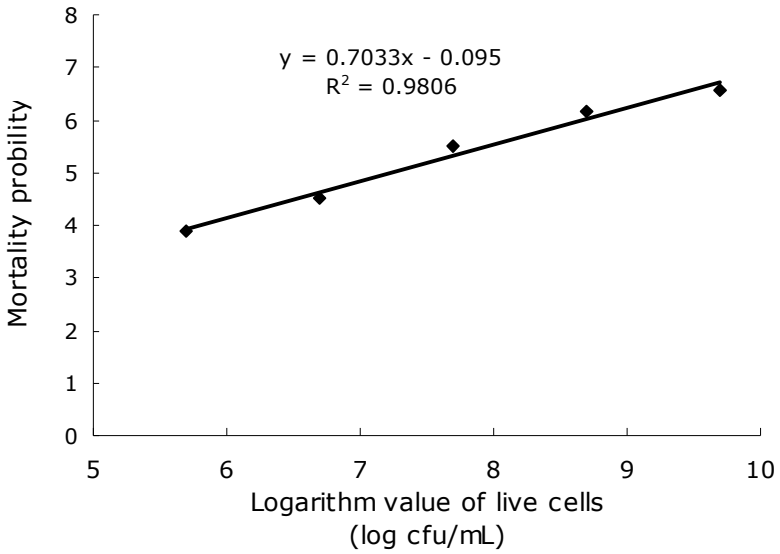

Figure 5. Correlation curve and regression equation of the strain W10's live cell concentration and mortality.

environment and is an opportunistic human pathogen, well known as a food pollutant (Andreeva et al., 2007). It often causes diarrhoeic and emetic food poisoning outbreaks (De Santis et al., 2008) and is characterized by the ability to produce several extracellular virulent hemolysins, which are able to cause erythrocyte lysis and is regarded as having a role in infections (Lund et al., 2000; Beecher and Wong, 2000; Baida and Kuzmin, 1996; Kotiranta et al., 2000). In this study, we found that *B. cereus* strain W10 could produce hemolysins for its ability to form hemolytic rings on RBA plates (Figure 1). Thus, *B. cereus* strain W10 is a potential threat to the safety of tiger frog food.

To our knowledge, no reports are available about the tiger frog disease infected by *B. cereus*. However, the tiger frogs challenged with *B. cereus* strain W10 displayed the signs of apparent dull black liver, as well as hemorrhage on the head and mouth corner in the present study, which indicated that *B. cereus* W10, may be a potential pathogen of healthy tiger frogs. Furthermore, the minimal infective dose of *B. cereus* in food poisoning is estimated to be 103 cfu/g of food (Pirhonen et al., 2005), although the minimal infective dose could hardly cause the death of healthy tiger frogs, according to the correlation curve and regression equation of the live cell concentration and mortality (Figure 5), this dose could potentially cause infection in humans.

In conclusion, a virulent strain W10 of *B. cereus* was identified and characterized to be a potential pathogen for the cultured tiger frogs. As a pathogen of zoonotic diseases, the ubiquitous distribution of the virulent *B. cereus* strain may be a threat to the safety of the cultured tiger frog food for its ability to produce endospores and enterotoxins. Therefore, virulent *B. cereus* strain should be paid more attention to in cultured tiger frog products.

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