Preplanned Studies

Isolation and Identification of *Burkholderia gladioli* pathovar *cocovenenans* from Black Fungus and Characteristics of the *Bon* Gene Cluster — Shanghai Municipality, China, 2023

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Summary

What is already known about this topic?

Burkholderia gladioli (*B. gladioli*) pathovar *cocovenenans* (*BGC*), a foodborne pathogen, can cause lethal poisoning. Most cases have been reported in China, primarily originating from fermented cereal products.

What is added by this report?

This study investigated the prevalence of *BGC* contamination in commercially available fungi and analyzed the environmental conditions for bongkrekic acid (BA) production in Shanghai. The overall detection rate of B. gladioli in the 85 samples was 44.7% . The highest detection rate was 94.4%, in dried black fungus, followed by fresh *Tremella fuciformis* (*T. fuciformis*) with 16.6%, fresh black fungus with 9.1%, and dried T. fuciformis with 3.8%. BGC was detected only in dried black fungus, with a detection rate of 39% . The results of this study demonstrate that all *BGC* strains carry the *bon* gene cluster encoding BA, indicating that *bonABCDFGHIJKLM* plays an essential role in the biosynthesis of BA.

What are the implications for public health practice?

Diagnostic polymerase chain reaction methods could enable rapid identification of BA-producing *BGC*, providing a potential clinical risk marker. People should avoid eating fungi products soaked 24 hours or more, no matter the temperature.

ABSTRACT

Objective: *Burkholderia gladioli* (*B. gladioli*) pathovar *cocovenenans* (*BGC*), a foodborne pathogen, can cause lethal poisoning. Most cases have been reported in China, primarily originating from fermented cereal products. In this study, we investigated the prevalence of *BGC* contamination in commercially available fungi and analyzed the environmental conditions for bongkrekic acid (BA) production in Shanghai. BA testing and animal experiments were conducted to confirm the relationship between *bon* genes and BA biosynthesis, and to clarify the causes of poisoning.

Methods: The association between the *bon* gene cluster and BA synthesis was analyzed through whole-genome sequencing and animal testing to identify the gene cluster responsible for BA synthesis.

Results: The overall detection rate of *B. gladioli* in the 85 samples was 44.7% (38/85). The highest detection rate was in dried black fungus (94.4%; 34/36), followed by fresh *Tremella fuciformis* (*T. fuciformis)* (16.6%; 2/12), fresh black fungus (9.1%; 1/11), and dried *T. fuciformis* (3.8%; 1/26). *BGC* was detected only in dried black fungus, with a detection rate of 39% (14/36). In the crude extract solutions obtained from the 14 *BGC* cultures, BA concentrations ranged from 0.33 μg/mL to 714.83 μg/mL. Both the crude extract solution and the ten-fold concentrated solution caused death in mice.

Conclusion: The results of this study demonstrate that all *BGC* strains carry the *bon* gene cluster encoding BA, indicating that *bonABCDFGHIJKLM* plays an essential role in the biosynthesis of BA.

Burkholderia gladioli pv. *cocovenenans* (*BGC*) is a foodborne pathogen that can cause lethal food poisoning (*1*). Symptoms include abdominal pain, diarrhea, vomiting, weakness, and palpitations. From 2005 to 2020, 30 foodborne *BGC* disease outbreaks were reported in China, resulting in 85 deaths. These outbreaks were primarily caused by fermented cereal products in rural areas (*2*–*3*). A few outbreaks were also attributed to nonfermented foods. *BGC* causes poisoning by producing bongkrekic acid (BA), which blocks the mitochondrial adenine nucleotide

translocator and prevents respiratory chain phosphorylation (*4*–*5*). BA is thermal-stable and cannot be destroyed by cooking (*4*), thus posing a significant risk to food safety. Several factors influence *BGC* growth and BA production (*6*–*7*). Previous research has shown that the optimal temperature and pH for *BGC* growth are 37 °C and 6.0, while the optimal conditions for BA production are 30 °C and pH 7.0 (*8*).

In this study, we investigated the rate of *BGC* contamination in commercially available fungi and analyzed the environment for BA production in Shanghai. BA testing and animal testing were conducted to confirm the relationship between *bon* genes and BA biosynthesis and to identify the mechanism of poisoning. Recommendations for policymakers are also provided based on our findings.

A total of 85 fungal food samples were collected from Shanghai markets in 2023. 60 samples were purchased from markets in Xuhui, Changning, and Minhang Districts, while 25 were acquired from online shops in Shanghai. The samples included 36 dried black fungus, 11 fresh black fungus, 26 dried *T. fuciformis*, and 12 fresh *T. fuciformis*. Fresh black fungus and fresh *T. fuciformis* were tested directly. Dried black fungus and dried *T. fuciformis* were soaked in sterile water for 24 h, 48 h, and 72 h at 4 °C, 26 °C, and 37 °C. All samples were analyzed according to the National Food Safety Standard-Food Microbiology Inspection for Burkholderia gladioli (Pseudomonas cocovenenans subsp. farino fermentans) (GB4789.29-2020).

High-performance Liquid Chromatography (HPLC) was used to quantify the amount of BA in the soaking liquid and crude extracts of black fungus. The crude extracts were produced by growing black fungus on potato dextrose agar (PDA) plates. BA soaking liquid was filtered through a 0.22 μm filter and used to determine the BA concentration according to the National Food Safety Standard-Determination of Bongkrekic Acid in Food (GB5009.189-2016). The mobile phases consisted of water and formic acid with a flow rate of 1 mL min^{-1}. Analysis time was 20 min, and the column temperature was set to 30 °C. *B. gladioli* that can metabolize Bongkrekic acid is defined as *BGC*.

The crude extract at a low dosage of 235.1 μg/mL was concentrated 10 times using a rotary evaporator to develop a high-dosage crude extract. Kunming mice (Shanghai Jiesijie Laboratory Animal Co.) weighing 18g were administered 0.5 mL of low-dosage or high-dosage crude extract intragastrically (three mice per group), followed by 7 days of observation.

All 38 *B. gladioli* isolates and the reference strain (CICC 25108) underwent short-read sequencing. Genomic DNA was extracted using a DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol (prelyzing the cells with lysozyme, 100 mg/mL, for 30 min). The concentration, quality, and integrity of the extracted DNA were determined using a Qubit Fluorometer (Thermo Scientific Waltham, MA, USA). Subsequently, genomic DNA was sent to the facility for next-generation short-read sequencing on an Illumina HiSeq platform (Illumina, USA).

The BA biosynthetic gene cluster *bon* (GenBank Accession JX173632) from *BGC* DMSZ11318 was used as the reference sequence. SeqMan (Lasergene 7.1) was used to extract the nucleotides of the *bon* gene cluster from the assembled sequences. MegAlign (Lasergene 7.1, DNASTAR, Inc. Madison, USA) and BioEdit (version 7.0.9.0, BIOEDIT LIMITED, Manchester, UK) were used to align and analyze the nucleotide identity of the *bon* genes.

The associations among the detection rates of *B. gladioli* isolates and *BGC* isolates under different conditions were analyzed, including different soaking durations and temperatures.

The total detection rate of *B. gladioli* in the 85 samples was 44.7% (38/85). The highest detection rate was observed in dried black fungus (94.4%; 34/36), followed by fresh *T. fuciformis* (16.6%; 2/12), fresh black fungus (9.1%; 1/11), and dried *T. fuciformis* (3.8%; 1/26). *BGC* was detected exclusively in dried black fungus, with a detection rate of 39% (14/36). The detection rates of *B. gladioli* varied under different conditions (Table 1). No significant differences were observed among the detection rates of *B. gladioli* from samples soaked at 4 °C, 26 °C, and 37 °C for 24, 48, and 72 h. However, the detection rate of *B. gladioli* in unsoaked samples was higher than in samples soaked for 72 h at 26 °C and 37 °C. The detection rate of *BGC* was highest after soaking at 4 °C for 72 h. No *BGC* isolates were recovered from fresh *T. fuciformis* or fresh black fungus.

The results showed that BA concentrations in crude extracts prepared under identical conditions varied remarkably among isolates. The lowest BA concentrations were 0.33 μg/mL and 2.08 μg/mL, produced by *BGC*2347 and *BGC*2358, respectively. The highest BA concentration reached 714.83 μg/mL, with an average concentration of 303.16 μg/mL. These

TABLE 1. Detection rates of *B. gladioli* and *BGC* in black fungus after soaking in several conditions.

Detection rate species	Duration/temperature									
	0 h (%)	24 h, 4 ℃ (%)	24 h, 26 ℃ (%)	24 h, 37 ℃ (%)	48 h, 4 ℃ (%)	48 h, 26 ℃ (%)	48 h, 37 ℃ (%)	72 h, 4 ℃ (%)	72 h, 26 ℃ (%)	72 h, 37 ℃ (%)
B. gladioli	42.1 (16/38)*	26.3 (10/38)	26.3 (10/38)	15.8 (6/38)*	39.5 (15/38)	42.1 (16/38)	36.8 (14/38)	50 (19/38)*	18.4 (7/38)*	15.8 (6/38)*
BGC	15.8 (6/38)	7.9 (3/38)†	7.9 (3/38)†	7.9 (3/38)†	0	0	0	26.3 (10/38)†	2.6 (1/38)†	2.6 (1/38)†

Note: $P<0.05$ was regarded as statistically significant.
Abbreviation: *B. gladioli=Burkholderia gladioli; BGC=Burkholderia gladioli* pv. *cocovenenans*.
* Significant difference ($P<0.05$) between the detection rates of *B. gladioli* in different conditions.
† Significant difference ($P<0.05$) between detection rates of *BGC* in different conditions.

findings indicate that *BGC* isolates possess varying capabilities for BA production. In this study, two *BGC* isolates with BA concentrations of 0.33 μg/mL and 2.08 μg/mL in crude extracts were classified as low toxin producers, while the remaining isolates were considered high toxin producers (Figure 1). No BA was detected in the soaked fungus liquid.

The crude extracts and their 10-fold concentrated versions (235.1 μg/mL and 2351 μg/mL, respectively) were used for animal testing. Mice were administered 0.5 mL of BA extract at these two concentrations via intragastric gavage. The final dosages per mouse (18 g) were 6.43 mg/kg and 64.3 mg/kg, respectively. Symptoms began to appear 2 hours after administration. All six mice (across both treatment groups) exhibited symptoms including raised hair, restlessness, and atrophy, followed by staggering, limb paralysis, and hesitation. Four hours after administration, all three mice in the high-dosage group died, and after 6 hours, two mice in the low-dosage group died. The remaining mice continued to show symptoms. Observation continued for 7 days, during which the surviving mouse gradually recovered to normal condition. Meanwhile, mice in the control group maintained a normal diet and remained healthy (Figure 1).

In total, 14 of 34 *B. gladioli* isolates were found to harbor *bonABCDFGHIJKLM*, and these isolates were identified as *BGC*. The concentration of BA produced by these isolates ranged from 0.33 μg/mL to 714.83 μg/mL. BA testing revealed that *BGC* isolates harboring *bonABCDFGHIJKLM* produced BA, whereas those without *bonABCDFGHIJKLM* did not produce BA.

DISCUSSION

Controlling contamination in raw materials is crucial for preventing *B. gladioli* contamination in dried black fungus and *T. fuciformis*. In this study, the detection rate of *B. gladioli* isolates reached 94.4% in dried black fungus, whereas it was only 3.8% in dried *T. fuciformis*, suggesting that *T. fuciformis* has a substantially lower risk of *B. gladioli* contamination. The detection rate of *BGC* isolates was 39%, higher than the 9.4% previously reported in Guangdong, China (*9*). This higher detection rate can be attributed to our use of both Mannitol yolk polymyxin (MYP) agar plates and PDA plates, whereas GB4789.29-2020 recommends only PDA. Our results demonstrated that MYP plates provided better selectivity and specificity for *B. gladioli* isolates.

To simulate the pre-consumption soaking process of dried black fungus and *T. fuciformis*, samples were soaked in sterilized water at different temperatures for varying durations. Notably, no BA was detected in any of the soaking fungus liquid samples. These results suggest that sterilized water does not provide optimal conditions for BA biosynthesis in black fungus. Even when soaked at 37 °C for 72 h, no BA was detected, indicating that soaking black fungus and *T. fuciformis* directly in sterile water is safe as it does not promote BA production.

Intragastric administration in mice confirmed that BA crude extracts could cause disease and death. The levels of toxin production were verified by BA concentration assay. These results provided a foundation for analyzing the relationship between *bon* gene clusters and BA biosynthesis. We analyzed the diversity of *bon* gene clusters and verified the predicted functions of different *bon* genes by measuring BA concentrations using HPLC of culture extracts and conducting animal tests. All 14 *BGC* isolates carrying *bonABCDFGHIJKLM* produced BA. Cerith et al. reported that *BGC* isolates produced varying amounts of BA *in vitro* (*10*). Furthermore, the 24 *B. gladioli* isolates in this study that lacked *bonABCDFGHIJKLM* did not produce BA, confirming the essential role of *bonABCDFGHIJKLM* in BA biosynthesis, which aligned with previous findings (*11*). The nucleotide

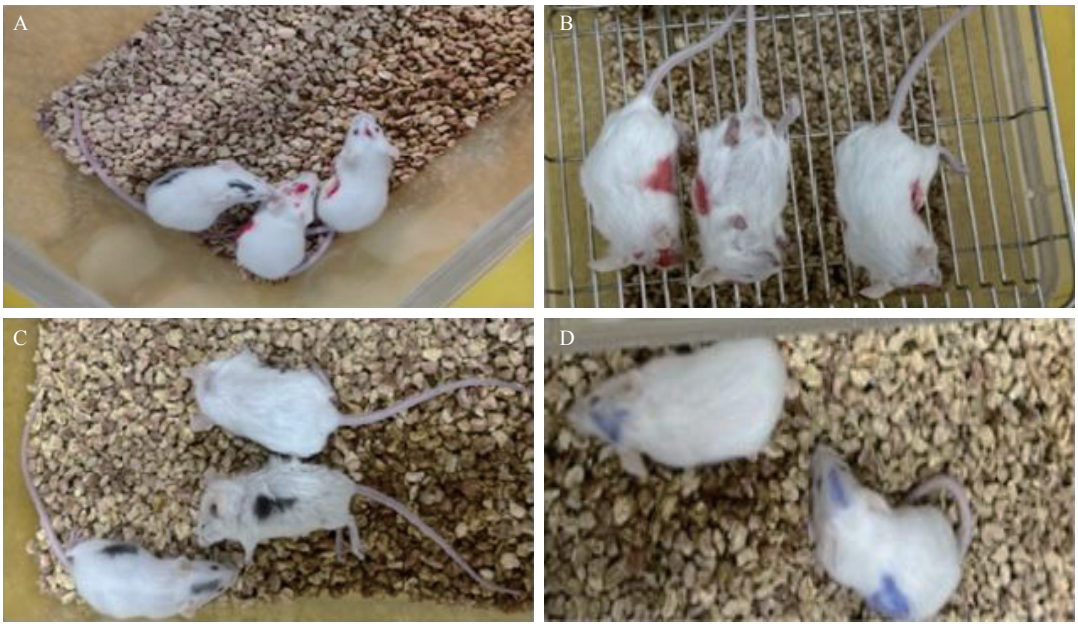

FIGURE 1. Intragastric administration of crude extracts in mice. (A) All mice were normal before intragastric administration. (B) All three mice died within 4 hours of high-dosage administration. (C) Two of three mice died within 6 hours of low-dosage crude extract administration. (D) All mice in the control group remained normal after 7 days on a standard diet.
Note: The mice labeled blue were the control group (not injected), those labeled black received low-dosage administration, and red indicated high dosage. For (A) The remaining mouse survived but exhibited symptoms including raised hair, restlessness, and weight loss compared to the control group.

alignment of *bonABCDFGHIJKLM* revealed considerable variation in identity, especially compared to the reference strain DMSZ113811. However, no obvious differences in *bonABCDFGHIJKLM* identity were observed between high-virulence and low-virulence isolates compared to the reference strain. This study suggests that diagnostic polymerase chain reaction methods could enable rapid identification of BA-producing *BGC*, providing a potential clinical risk marker.

This study could be enhanced in several ways. First, the potential involvement of other regulatory genes in the expression of *bonABCDFGHIJKLM*, warrants further investigation. Second, the results should be verified through additional cases and samples. Finally, potential errors in gene testing may have influenced our findings, suggesting that more reliable gene testing methods should be adopted in subsequent studies.

Conflicts of interest: No conflicts of interests.

Ethical statement: The animal study protocol was approved by the Institutional Review Board (Ethics Committee) of the Shanghai Municipal Center for Disease Control and Prevention (protocol code CH20230036, 2023.3.27).

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