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Phenotypic and genotypic screening of *Bacillus cereus* local isolates for their antimicrobial resistance

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Abstract. *Farhani I, Dewanti-Hariyadi R, Lioe HN. 2024. Phenotypic and genotypic screening of* Bacillus cereus *local isolates for their antimicrobial resistance. Biodiversitas 25: 4507-4514. Bacillus cereus* is a sporeformer bacterium that ranks second as Indonesia's most common cause of foodborne illnesses from 2000 to 2015. The bacterium produces cereulides and enterotoxins, which trigger emetic and diarrheal syndromes. The rise of antibiotic resistance in pathogens such as *B. cereus* could complicate treatment and allow the bacterium to act as the reservoir of antibiotic-resistant genes. Currently, antibiotic resistance in *B. cereus* has not been reported in Indonesia. This study aims to evaluate the antibiotic resistance of 21 *B. cereus* local isolates obtained from foods against eight different antibiotics. The phenotypic resistance was evaluated using the Kirby-Bauer disc diffusion method, while resistance genes were detected using a polymerase chain reaction targeting the *bla*1, *tetL*, and *tet*B genes. None of the isolates (0%; 0/21) showed resistance to chloramphenicol, ciprofloxacin, or erythromycin. However, all (100%; 21/21) were resistant to ampicillin, cefoxitin, cephalothin, and penicillin G. Additionally, 4.8% (1/21) were susceptible to erythromycin, while 33.3% (7/21) were resistant to tetracycline. The detection of resistance-encoding genes revealed that 100% (21/21) of the isolates possessed the *bla*1 gene, but *tetL and tetB* genes were absent in any of the isolates (0%; 0/21).

Keywords: Antibiotic resistance, *Bacillus cereus*, PCR analysis, resistance genes, screening

Abbreviations: MHA: Mueller Hinton Agar; NFW: Nuclease Free Water; PCR: Polymerase Chain Reaction; TSA: Tryptic Soy Agar; TSB: Tryptic Soy Broth

INTRODUCTION

Foodborne outbreaks are becoming more severe due to globalization and active food trade among countries (Lynn et al. 2014). Globally, there are 600 million cases of foodborne diseases, with 420,000 deaths yearly due to unsafe food consumed. The World Health Organization (WHO) studied 31 foodborne hazards and bacteria (226,526,634 cases), which are the most common causative agents as compared to viruses (138,513,782 cases) and parasites (10,284,561 cases) (Havelaar et al. 2015; Lee and Yoon 2021). One of the major foodborne pathogen is *Bacillus cereus*. The European Food Safety Authority (EFSA) and the Center for Disease Control (CDC) stated that the demands of consumers for complex and mildly processed foods with minimal refrigerated shelf life are driving the increase of *B. cereus* outbreaks worldwide (Rodrigo et al. 2021).

Bacillus cereus is a rod-shaped, Gram-positive bacteria that can grow both aerobically and anaerobically. Commonly found in soil, they could contaminate food and crops easily. Due to their high adaptability, they survive in environments with pH levels from 4.5 to 9.5, water activity levels as low as 0.93, and temperatures ranging from 4°C (for psychotropic strains) to 48°C. They can also withstand salt concentrations up to 70% (Duport et al. 2016). Consumption of food containing $>10^5$ *B. cereus* cells/g can lead to food poisoning (Hwang and Park 2015). The vegetative cells produce heat-labile enterotoxins in the small intestine, causing diarrheal illness, while germinated spores may produce cereulide toxin in food, leading to emetic illness (Ellouze et al. 2021). Its endospores are highly resilient, surviving various stresses and extreme conditions, allowing widespread distribution in nature. Their hydrophobic spores adhere, allowing them to stick to surfaces, germinate, and increase in processing equipment. Diarrheal and emetic syndromes typically appear 8-16 hours and 1-5 hours after ingesting contaminated food, respectively (Merzougui et al. 2014; Messelhäusser et al. 2014). In 2019, the European Union stated that *B. cereus* was the most frequently reported cause of foodborne illness, with 155 episodes, 1.636 cases, 44 hospitalizations, and seven deaths (Kavanaugh et al. 2022). However, in Indonesia, *B. cereus* ranked second as the most common cause of outbreaks between 2000 and 2015, accounting for 34 episodes (19.4% of cases) out of a total of 175 (Arisanti et al. 2018). The latest case was linked with contaminated chicken satay (A southeast Asian dish consisting of pieces of chicken meat on a stick, served with peanut sauce) in Yogyakarta, causing 188 villagers to have several predominate symptoms, such as diarrhea, nausea and abdominal cramps (Son et al. 2020).

According to the World Health Organization (WHO), one of the significant health issues of the 21st century is the emergence and spread of antibiotic resistance, which has

been the topic of several surveys. *B. cereus*, also known as *B. cereus* sensu stricto, is frequently resistant to penicillin and other β-lactam antibiotics, and it has the potential to develop resistance to widely used antibiotics, including ciprofloxacin, cloxacillin, erythromycin, tetracycline, and streptomycin (Fiedler et al. 2019; FAO 2021). Although most foodborne illnesses caused by the *B. cereus* group do not typically require antibiotic treatment, these bacteria are becoming increasingly concerning due to their expanding role in gastrointestinal disorders and other serious infections, particularly among immunocompromised patients. These infections are predominantly seen in newborns, the elderly, and individuals with weakened immune systems, but they can also occur in healthy individuals. The spectrum of infections includes localized skin and wound infections, especially post-operative ones, as well as more severe conditions such as septicemia (blood poisoning), meningitis (inflammation of the membranes around the brain and spinal cord), pneumonia (lung infection), and endocarditis (infection of the heart valves). The increasing incidence and severity of these infections highlight the importance of monitoring and addressing *B. cereus* as a significant pathogen (Dietrich et al. 2021).

The extent to which *B. cereus* group strains serve as a source of transferable antibiotic resistance genes in the food chain remains poorly understood. This study aims to evaluate the antimicrobial resistance characteristics of local *B. cereus* isolates by screening for resistance to various antibiotic classes and identifying the presence of genes associated with antibiotic resistance.

MATERIALS AND METHODS

Culture preparation

Inoculum preparation

Twenty-one local isolates of *B. cereus* were previously isolated from white pepper (Nanteza et al. 2022a), chilli (Nanteza et al. 2022b), cooked white rice (Rizki et al. 2022), fresh vegetables (green bean, shallot, potato, and water spinach), and pasteurized and raw milk was grown in sterile TSB (Oxoid™, UK) for 24 h at 36±1°C to achieve its exponential phase. The stock culture was prepared by looping in the *B. cereus* culture and streaking it into TSA (Oxoid™, UK). Then, it was incubated for 24 h at 36±1°C. After being incubated, the culture was stored at 4°C until further use. The working cultures (or inoculum) were prepared by transferring 1 mL of overnight culture into 9 mL of sterile TSB, and then incubated for 24 h at 36±1°C. Another way to prepare is by taking a loopful of *B. cereus* culture into 9 mL of sterile TSB, vortexed, and incubated for 24 h at 36±1°C (Albaridi and Yehia 2022).

Biochemical confirmation of B. cereus *isolates*

All *B. cereus* local isolates underwent a biochemical confirmation test before further use to ensure no contaminant was present. The test was done using Gram staining, spore staining, and catalase test following the procedure described in the FDA's Bacteriological Analytical Manual (BAM). For Gram and spore staining, both vegetative cells and spores' physical characteristics were observed under a microscope (Olympus CX21, Japan) (Nanteza et al. 2022b).

Antibiotic resistance screening

The test was done using the Kirby-Bauer disc diffusion method following the M100 Clinical and Laboratory Standards Institute (CLSI) guidelines for *Staphylococcus aureus* (CLSI 2020; Fraccalvieri et al. 2022) A total of eight antibiotics (Oxoid™, UK) were tested, including ampicillin (AMP, 10µg), penicillin G (P, 10 U), Cephalothin (KF, 30 µg), cefoxitin (FOX, 30 µg), erythromycin (E, 15 µg), chloramphenicol (C, 30 µg), tetracycline (TE, 30 µg), ciprofloxacin (CIP, 5 µg). The isolates were prepared to a concentration of 10^8 CFU/mL (equivalent to an optical density of 0.5 McFarland standards measured at 620 nm) and swabbed onto MHA (Oxoid™, UK) using a sterile cotton swab. The antimicrobial discs were placed onto the inoculated agar within 15 min of inoculation using sterile forceps. A maximum of 4 discs were applied on each agar plate to avoid overlaps in zones of inhibition that could implicate the accuracy of zone measurement. After incubation of 18 h at 36±1°C, the inhibition zones were measured and interpreted by referring to CLSI guidelines for *S. aureus*. *S. aureus* strain ATCC 25923 was used as a quality control strain.

Detection of antibiotic resistance genes using PCR

DNA extraction

Isolates with high inhibition test results counted as resistance were subcultured on TSB and incubated for 24 h at 36±1°C. Extraction and purification of the genomic DNA of *B. cereus* isolates were done using the Presto™ Mini gDNA Bacteria Kit (Geneaid) following the manufacturer's instructions (Geneaid 2017). Briefly, 1.5 mL of the incubated culture was transferred into sterile 2 mL microcentrifuge tubes and centrifuged at 14000× g for 60 s. A mixture containing lysozyme and Gram+ buffer was added to each sample, vortexed, and incubated at 37°C for 30 min. Proteinase K was added and incubated at 60°C for at least 10 min. Cell lysis was done by adding GB buffer followed by incubation at 70°C for at least 10 minutes. It was followed by the addition of 5 µL RNAse A 10 mg/mL (ThermoFisher Scientific) with vigorous shaking and incubation at 25°C for 5 min, as well as the addition of absolute ethanol. The mixture was transferred to a GD Column and centrifuged at 14000 ×g for 2 mins. The DNA was washed with W1 buffer and wash buffers and eluted with the addition of a pre-heated elution buffer. The DNA purity and concentration were measured using NanoPhotometer® NP80 (Implen, Germany) at 260 and 280 nm. The purified DNA was stored at -20°C prior to PCR amplification.

DNA amplification

The antibiotic-resistant encoding genes were detected through DNA amplification using the PCR thermal cycler 2720 (Applied Biosystems, USA). PCR product of a 680 (bp) sequence in the *bla*1 gene was detected using the following primers: (5'-CATTGCAAGTTGAAGCGAAA-3') as

forward primer and (5'-TGTCCCGTAACTTCCAGCTC-3') as reverse primer. The running conditions are as follows: started with an initial denaturation at 94°C for 5 mins, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 40 s, extension at 72°C for 45 sec, and final extension at 72°C for 10 min (El-Tawab et al. 2020; Algammal et al. 2024). For PCR product of a 267 (bp) sequence in the *tet*L gene detected using the forward primer sequence of (5'-TCGTTAGCGTGCTGTCATTC-3') and reverse primer of (5'-GTATCCCACCAATGTAGCCG-3'). Running condition was started with an initial denaturation at 94°C for 5 mins, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, extension at 72°C for 1 min, and final extension at 72°C for 10 min (Saeed et al. 2018). Lastly, the *tet*B (723 bp) gene was detected using the forward primer of (5'-CCCAGTGCT GTTGTTGTCAT-3') and reverse primer of (5'-CCACCACCAGCCAATAAAAT-3'). Running condition was started with an initial denaturation at 95°C for 10 mins, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 7 min (Sik and Akan 2024).

The PCR mixture was: GoTaq® Green Master Mix (Promega, USA) (12.5 µL for *bla*1 and *tet*L and 10 µL for *tet*B), primer set of each gene (Thermo Scientific, USA) (1 µL of reverse and 1 µL of forward primer), template DNA (6 µL for *bla*1; 5 µL for *tet*L; 1 µL for *tet*B), and Nuclease Free Water (NFW) (Thermo Scientific, USA) until total volume was 25 µL (Saeed et al. 2018; El-Tawab et al. 2020; Algammal et al. 2024).

Visualization with agarose gel electrophoresis

PCR products were separated on 2% agarose gel through electrophoresis (Bio-Rad Laboratories-Segrete, Italy) for 45 min at 90 V. A 100 (bp) DNA ladder (Thermo Fisher Scientific, USA) was used as a marker, and the negative control was NFW. The gels were visualized by gel documentation (Bio-Rad Laboratories-Segrete, Italy) with GelRed® (Biotium, USA) as a gel staining agent (Saeed et al. 2018; Nanteza et al. 2022a).

RESULTS AND DISCUSSION

Biochemical confirmation of *B. cereus* local isolates

Biochemical confirmation of all isolates was done to ensure no contaminant was present. Gram staining was performed to differentiate between Gram-positive and Gram-negative bacteria, coloring their vegetative cells either red (Gram-negative) or violet (Gram-positive). This technique also identifies bacteria's morphological structures. All 21 isolates tested showed rod-shaped, purple cells under the microscope, indicating they are Gram-positive like *B. cereus* (Figure 1.A). Spore staining was done to distinguish the spores from vegetative cells. The positive result of all isolates from spore staining reveals green spores and red vegetative cells, confirming the bacteria's spore-forming nature (Figure 1.B). The catalase test aimed to distinguish catalase-producing bacteria from non-producers. In this study, the test confirmed that the isolates are catalase-positive. All isolates exhibited bubble formation, indicating catalase presence (Figure 1.C).

Antibiotic susceptibility testing

All isolates (100%; 21 out of 21) exhibited resistance to β-lactam class antibiotics, consisting of ampicillin, cefoxitin, cephalothin, penicillin G, and erythromycin, yet none of them showed resistance to chloramphenicol, ciprofloxacin, and erythromycin. Interestingly, various results were obtained from the tetracycline results. It was found that 8 out of 21 isolates (38.1%) were susceptible, 6 out of 21 isolates (28.6%) were intermediate, and 7 out of 21 isolates (33.3%) were resistant to tetracycline (Table 1).

To further understand the resistance mechanisms, isolates with the highest levels of resistance (β-lactams) and variable resistance data (tetracycline) were subjected to genetic screening to detect antibiotic resistance genes.

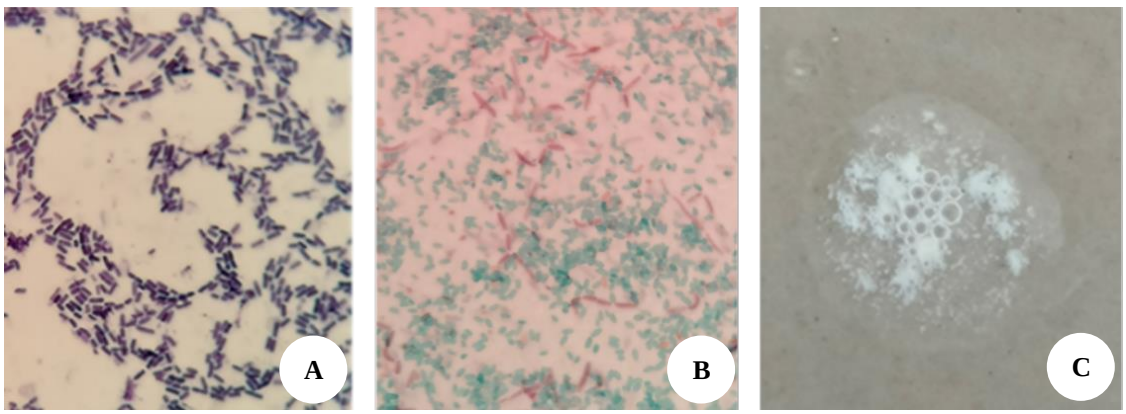

Figure 1. Example of A. Gram staining; B. Spore staining; C. Catalase test positive results of the isolates indicated by violet-colored vegetative cells, green-colored spores, and bubble formation, respectively. For Gram and spore staining, results were subjected to microscopic observation under 1000x magnification

Detection of antibiotic resistance genes using PCR method

The resistance properties identified in the earlier phenotypic screening were subsequently confirmed by detecting encoding genes using PCR techniques. DNA extraction is the initial step of gene detection using the PCR method. Following the manufacturer's instructions, this study used the Presto™ Mini gDNA Bacteria Kit (Geneaid). The purity and concentration of extracted DNA were assessed using a Nanodrop 2000 spectrophotometer at 260 and 280 nm. The ratio in the range of 1.8-2.0 was used as it counted as a pure extract.

This study selected three target genes: *bla*1, *tet*L, and *tet*B, corresponding to resistance observed in β-lactam and tetracycline antibiotic classes. The results of detecting the antibiotic-resistant genes, i.e., *bla*1, *tet*L, and *tet*B, are presented in Figures 2, 3, and 4 respectively.

Table 1. Antibiotic susceptibility of *Bacillus cereus* local isolates (n = 21) towards eight kinds of antibiotics

Antibiotic classes	Antibiotics	Number of isolates (%)		
		Susceptible	Intermediate	Resistant
β-lactam	Ampicillin (AMP)	0 (0)	0 (0)	21 (100)
	Penicillin G (P)	0 (0)	0 (0)	21 (100)
	Cephalothin (KF)	0 (0)	0 (0)	21 (100)
	Cefoxitin (FOX)	0 (0)	0 (0)	21 (100)
Macrolides	Erythromycin (E)	1 (4.8)	20 (95.2)	0 (0)
Phenicols	Chloramphenicol (C)	21 (100)	0 (0)	0 (0)
Tetracyclines	Tetracycline (TE)	8 (38.1)	6 (28.6)	7 (33.3)
Fluoroquinolones	Ciprofloxacin (CIP)	21 (100)	0 (0)	0 (0)

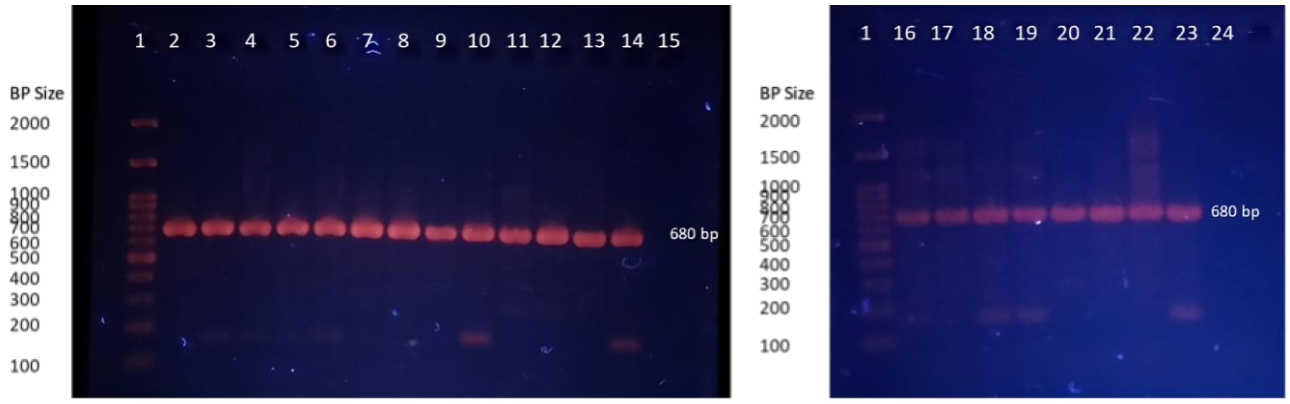

Figure 2. Visualization of *bla*1 gene bands of *Bacillus cereus* on agarose gel. Lane 1: 100 (bp) ladder; Lane 2-4: White Pepper isolates; Lane 5-7: Chilli isolates; Lane 8-11: Pasteurized milk isolates; Lane 12-13: Raw milk isolates; Lane 14: Cooked white rice isolates; Lane 16-19: Cooked white rice isolates; Lane 20: Green bean isolate; Lane 21: Onion isolate; Lane 22: Potato isolate; Lane 23: Water spinach isolate; Lane 15 and 24: NFW (negative control)

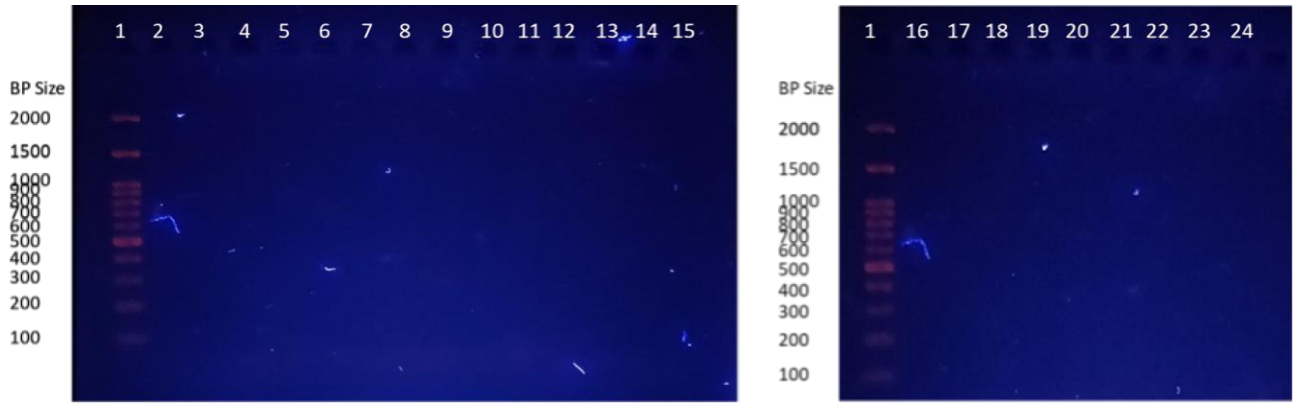

Figure 3. Visualization of *tet*L gene bands on an agarose gel. Lane 1: 100 (bp) ladder; Lane 2-4: White Pepper isolates; Lane 5-7: Chilli isolates; Lane 8-11: Pasteurized milk isolates; Lane 12-13: Raw milk isolates; Lane 14: Cooked white rice isolates; Lane 16-19: Cooked white rice isolates; Lane 20: Green bean isolate; Lane 21: Onion isolate; Lane 22: Potato isolate; Lane 23: Water spinach isolate; Lane 15 and 24: NFW (negative control)

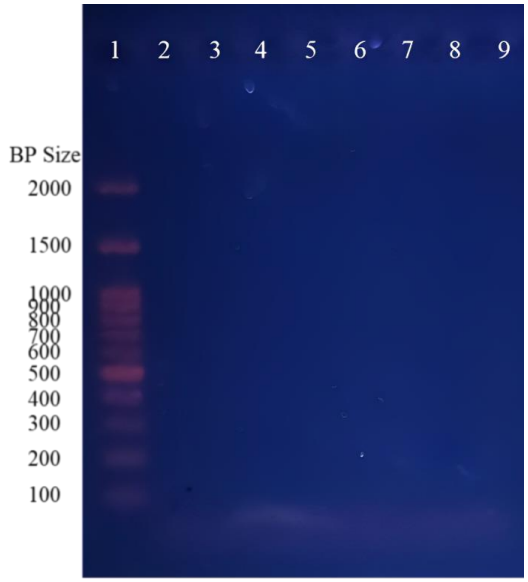

Figure 4. Visualization of *tet*B gene bands of *Bacillus cereus* isolates exhibiting tetracycline resistance was done by using the Kirby-Bauer test on an agarose gel. Lane 1: 100 (bp) ladder; Lane 2-3: Chilli isolates; Lane 4: Pasteurized milk isolate; Lane 5-6: Cooked white rice isolates; Lane 7: Potato isolate; Lane 8: Water spinach isolate; Lane 9: NFW (negative control)

All of the (100%; 21 out of 21) isolates examined exhibited distinct and strong bands corresponding to the target size of 680 (bp) for the *bla*1 amplicon (Figure 2). The findings of this result align with those reported in previous studies found in meat products (El-Tawab et al. 2020), ice cream (Fraccalvieri et al. 2022), fish (Algammal et al. 2022), plant foods and edible wild mushrooms (Cha et al. 2023)

The detection of tetracycline-resistant genes (*tet*L and *tet*B) in the local *B. cereus* exhibiting resistance to the antibiotic using the Kirby-Bauer test showed different results. Although some isolates exhibit tetracycline resistance by phenotypic assay, none of the 21 isolates show any bands at the target length of 267 (bp) for *tet*L, suggesting that no isolate possessed the *tet*L gene (Figure 3). Due to the absence of the *tet*L gene in all isolates, the tetB gene was detected only for the resistant isolates. Similar to the results with *tet*L, none of the isolates possessed the *tet*B gene (Figure 4), strongly suggesting that other genes encode the resistance.

Discussion

Bacillus cereus has a thick cell wall due to its peptidoglycan layers, which retain the violet stain. The staining process involved four steps: crystal violet dye, iodine mordant, alcohol decolorizer, and safranin counterstain. The crystal violet dye binds within the cell, forming a complex with iodine, which is retained despite the decolorization step (Paray et al. 2023).

Spores serve as a defense mechanism, shielding bacteria from harsh environments. The staining process requires agents capable of penetrating thick spore walls, such as the Schaeffer-Fulton method, utilizing Malachite Green and Saffranin dyes. Malachite Green, with a pH of 11.2, is soluble in water and alcohol, effectively staining bacteria due to its alkaline properties (Oktari et al. 2017). Catalase catalyzes the breakdown of hydrogen peroxide into water and oxygen, resulting in gas bubble formation. Typically, a 3% H_2O_2 solution is employed under aerobic conditions. Pathogens often produce catalase to counteract the host's immune response, utilizing hydrogen peroxide as a defensive agent, and to mitigate oxidative stress (Dong et al. 2022).

Antibiotics vary in their mechanisms of action and resistance. β-lactams disrupt bacterial cell wall synthesis by binding to penicillin-binding proteins (PBPs), activating autolytic enzymes, and causing cell lysis. Resistance occurs through reduced drug affinity to PBPs, enzymatic inactivation, or poor drug penetration. Erythromycin, chloramphenicol, and tetracycline inhibit protein synthesis by interfering with the translocation and peptide chain growth of the bacteria. Erythromycin resistance can arise from reduced cell membrane penetration or methylation of ribosomal RNA, decreasing drug affinity. Chloramphenicol resistance often involves a plasmid-encoded acetyltransferase that inactivates the drug. Tetracycline resistance typically involves plasmid-mediated reduction in drug accumulation. Ciprofloxacin inhibits DNA gyrase, affecting DNA replication, recombination, and repair, with resistance developing through efflux pumps or gyrase gene mutations (Vardanyan and Hruby 2006; Shariati et al. 2022).

Our findings align with previous studies on antibiotic resistance in *B. cereus* isolates, highlighting the bacteria's notable resistance to β-lactam antibiotics. These findings are consistent with previous studies examining antibiotic resistance in *B. cereus* isolates. *B. cereus* has been reported to exhibit considerable resistance to β-lactam antibiotics, a trend seen in various research efforts. For instance, a study by Fraccalvieri et al. (2022) on antimicrobial resistance in *B. cereus* group bacteria isolated from Italian-produced ice cream found that all tested *B. cereus* isolates exhibited complete resistance to ampicillin (100%), penicillin (97%), and cephalothin (94%). All *B. cereus* sensu lato strains were also sensitive to chloramphenicol (100%). Similarly, Jung et al. (2022) reported on the antibiotic susceptibility of *B. cereus* isolated from garlic chives in Korea, showing resistance to penicillin (100%) and cefoxitin (92.3%). Only one out of thirteen isolates (7.7%) showed intermediate resistance to cefoxitin, with intermediate resistance also detected for erythromycin (7.7%) and tetracycline (7.7%). In 2019, Fiedler et al. (2019) found that *B. cereus* showed widespread resistance to β-lactam antibiotics penicillin G (100%) and ampicillin (99.3%). Conversely, these strains were generally susceptible to ciprofloxacin (99.3%), chloramphenicol (98.6%), erythromycin (91.8%), and tetracycline (76.2%). These high susceptibility results towards chloramphenicol and ciprofloxacin align with the study by Kim et al. (2015) on strains of *B. cereus* isolated from Korean fermented soybean products, which were found fully susceptible to ciprofloxacin (100%) and chloramphenicol (100%). They also reported that the *B. cereus* isolates were susceptible to tetracycline (100%) but exhibited resistance to β-lactam antibiotics including ampicillin and penicillin

(100%). Overall, these studies consistently show that while *B. cereus* exhibits high resistance to β-lactam antibiotics; it remains generally susceptible to several other antibiotics, such as chloramphenicol and ciprofloxacin. Variations in percentage susceptibilities may result from multiple factors, including human behaviors such as the overuse and misuse of antibiotics, poor infection prevention measures, and a general lack of awareness. In the animal sector, the extensive use of antibiotics in livestock and aquaculture, as well as the transmission of resistant bacteria through food chains and direct contact with animals, plays a significant role. Environmental factors also contribute, with antibiotics and resistant bacteria being released into water bodies, soil, and waste systems. Additionally, wildlife interactions and habitat encroachment further facilitate the spread of resistant organisms (Endale et al. 2023).

Bacterial resistance to antibiotics arises from acquiring resistance genes found in both intracellular and extracellular DNA. Extracellular DNA derives from intracellular DNA, released through cell lysis of dead bacteria cells or active secretion of living bacterial cells. Transfer of resistance genes occurs through vertical and horizontal gene transfer between bacterial species. The persistence and mobility of these genes across environments play vital roles in the spread of antibiotic resistance. Intracellular genes occur in nutrient-rich conditions, while extracellular genes are prominent in aquatic environments. Extracellular genes can bind to soil and sediment, resist degradation, and endure longer than intracellular ones (Zarei-Baygi and Smith 2021).

The *bla* gene, known for providing resistance against β-lactam antibiotics, represents the largest category of antibiotic resistance genes, accounting for approximately 50-70% of reported antibiotic resistance, and is one of the prevalent antibiotic resistance genes observed in hospitals. Among the various types of habitats globally, including farms, cities, wastewater treatment plants, water bodies, soil, and air, *bla* genes rank second in prevalence as antibiotic-resistant genes. This gene is among Asia's top 10 antibiotic-resistance genes (Zhuang et al. 2021; Miao et al. 2022). This gene is plasmid-mediated, which means the transfer of the genes carried on plasmids facilitates their efficient spread within the microbiome. Nevertheless, detection of the *bla*1 gene can indicate the presence of β-lactamase genes, but it may not fully represent all *bla* genes. The *bla*1 gene is one specific type of β-lactamase gene, yet many different *bla* genes are present, each conferring resistance to various β-lactam antibiotics. It is essential to highlight that a thorough assessment of *bla* genes requires testing for multiple specific *bla* genes. Therefore, while identifying *bla*1 is helpful, it might not encompass a sample's entire range of β-lactamase genes.

There are some factors affecting the failure to detect a gene during PCR reaction followed by electrophoresis, such as depletion or thermal degradation of primers or nucleotides, thermal denaturation of the DNA polymerase, buildup of PCR product that interferes with DNA polymerase, reagent depletion, poor quality and/or contamination of template DNA, very high annealing temperature, extension time, presence of PCR inhibitors, and insufficient of DNA polymerase enzyme (Jansson and Hedman 2019). In this study, an optimization process was carried out to invalidate possible issues related to annealing temperature. Several running conditions have also been tried. In addition, the same DNA extract was also used in *bla*1 detection, and the result was positive.

The absence of the *tet*L gene from this study might be due to the tetracycline-resistant traits in these *B.cereus* isolates which were encoded by different *tet* genes since there are other types of *tet* genes. López et al. (2008) also reported a similar case where 23% of tetracycline-resistant isolates derived from honey did not carry any *tet* genes studied. In 2002, Agerso et al. (2002) also reported that 95.04% or 81 out of 88 tetracycline-resistant isolates showed no amplicons for any of the *tet* genes investigated. It is known that not all Gram-positive bacteria with phenotypic tetracycline-resistant traits possess any of the known *tet* genes examined (Roberts 1996). Interestingly, these findings are similar to Gram-negative bacteria in a study by Skockova et al. (2012), where 2 out of 37 tetracycline-resistant *Escherichia coli* isolates derived from raw cow's milk tested negative for any *tet* genes.

In conclusion, the prevalence of β-lactam antibiotic resistance among local *B. cereus* isolates is notably high, as indicated by consistent resistance phenotypically across all isolate sources. The presence of the *bla*1 gene further confirms this resistance, which serves as a representative marker. However, none of the *B. cereus* local isolates are resistant to chloramphenicol, ciprofloxacin, and erythromycin. Despite varied results for tetracycline resistance, it is still not sufficient to conclusively demonstrate resistance through *tet*L and *tet*B gene detection, as no isolates show having the tested genes. Other tetracycline-resistant genes (e.g., *tet*A, *tet*C, *tet*D, *tet*E, and *tet*K) should be investigated further to understand the mechanisms. In addition, a peptidomic analysis could offer valuable insights into bacterial peptide profiles, aiding in identifying resistance patterns to specific antibiotics.

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REFERENCES

Agerso Y, Jensen LB, Givskov M, Roberts MC. 2002. The identification of a tetracycline resistance gene tet (M), on a Tn 916-like transposon, in the *Bacillus cereus* group. FEMS Microbiol Lett 214 (2): 251-256. DOI: 10.1111/j.1574-6968.2002.tb11355.x

Albaridi NA, Yehia HM. 2022. The real role of select herb and spice extracts against *Bacillus cereus* ATCC 14579 growth in cooked rice. Food Sci Technol 42: e08521. DOI: 10.1590/fst.08521.

Algammal AM, Alfifi KJ, Mabrok M, Alatawy M, Abdel-moneam DA, Alghamdi S, Azab MW, Ibrahim RA, Hetta HF, El-Tarabili RM. 2022. Newly emerging MDR *B. cereus* in Mugil seheli as the first report commonly harbor nhe, hbl, cytK, and pc-plc virulence genes

and bla1, bla2, tetA, and ermA resistance genes. Infect Drug Resist 15: 2167-2185. DOI: 10.2147/IDR.S365254.

Algammal AM, Eid HM, Alghamdi S, Ghabban H, Alatawy R, Almanzalawi EA, Alqahtani TM, Elfouly SG, Mohammed GM, Hetta HF, El-Tarabili RM. 2024. Meat and meat products as potential sources of emerging MDR *Bacillus cereus*: groEL gene sequencing, toxigenic and antimicrobial resistance. BMC Microbiol 24 (1): 50. DOI: 10.1186/s12866-024-03204-9.

Arisanti RR, Indriani C, Wilopo SA. 2018. Kontribusi agen dan faktor penyebab kejadian luar biasa keracunan pangan di Indonesia: Kajian sistematis. Berita Kedokteran Masyarakat 34 (3): 99. DOI: 10.22146/bkm.33852. [Indonesian]

Cha X, Lin Y, Brennan C, Cao J, Shang Y. 2023. Antibiotic resistance of *Bacillus cereus* in plant foods and edible wild mushrooms in a province. Microorganisms 11 (12): 2948. DOI: 10.3390/microorganisms11122948.

CLSI [Clinical and Laboratory Standards Institute]. 2020. Performance Standards for Antimicrobial Susceptibility Testing, 30th Edition. CLSI supplement M100. Clinical and Laboratory Standards Institute, Wayne.

Dietrich R, Jessberger N, Ehling-Schulz M, Martlbauer E, Granum PE. 2021. The food poisoning toxins of *Bacillus cereus*. Toxins 13 (2): 98. DOI: 10.3390/toxins13020098.

Dong PT, Jusuf S, Hui J, Zhan Y, Zhu Y, Liu GY, Cheng JX. 2022. Photoinactivation of catalase sensitizes a wide range of bacteria to ROS-producing agents and immune cells. JCI Insight 7 (10): 1-19. DOI: 10.1172/jci.insight.153079.

Duport C, Jobin M, Schmitt P. 2016. Adaptation in *Bacillus cereus*: From stress to disease. Front Microbiol 7: 1550. DOI: 10.3389/fmicb.2016.01550.

Ellouze M, Silva NBD, Rouzeau-Szynalski K, Coisne L, Cantergiani F, Baranyi J. 2021. Modeling *Bacillus cereus* growth and cereulide formation in cereal-, dairy-, meat-, vegetable-based food and culture medium. Front Microbiol 12: 639546. DOI: 10.3389/fmicb.2021.639546.

El-Tawab AAA, Elhofy F, Shawky NA, Morsy DE. 2020. Molecular detection of antibiotic resistant bla gene in *B. cereus* isolated from meat products. Benha Vet Med J 38 (2): 152-155. DOI: 10.21608/bvmj.2020.27904.1199.

Endale H, Mathewos M, Abdeta D. 2023. Potential causes of spread of antimicrobial resistance and preventive measures in one health perspective-a review. Infect Drug Resist 16: 7515-7545. DOI: 10.2147/IDR.S428837.

FAO [Food and Agriculture Organization]. 2021. The FAO Action Plan on Antimicrobial Resistance 2021-2025. FAO, Rome. https://www.fao.org/documents/card/en/c/cb5545en/.

Fiedler G, Schneider C, Igbinosa EO, Kabisch J, Brinks E, Becker B, Stoll DA, Cho GS, Huch M, Franz CMAP. 2019. Antibiotics resistance and toxin profiles of *Bacillus cereus*-group isolates from fresh vegetables from German retail markets. BMC Microbiol 19 (1): 250. DOI: 10.1186/s12866-019-1632-2.

Fraccalvieri R, Bianco A, Difato LM, Capozzi L, Sambro LD, Simone D, Catanzariti R, Caruso M, Galante D, Normanno G, Palazzo L, Tempesta M, Parisi A. 2022. Toxigenic genes, pathogenic potential and antimicrobial resistance of *Bacillus cereus* group isolated from ice cream and characterized by whole genome sequencing. Foods 11 (16). DOI: 10.3390/foods11162480.

Geneaid. 2017. Presto TM Mini gDNA Bacteria Kit. Geneaid. https://www.geneaid.com/data/download/attached/160274590882278 4327.pdf.

Havelaar AH, Kirk MD, Torgerson PR, Gibb HJ, Hald T, Lake RJ, Praet N, Bellinger DC, Silva NRD, Gargouri N, Speybroeck N, Cawthorne A, Mathers C, Stein C, Angulo FJ, Devleesschauwer B. 2015. World health organization global estimates and regional comparisons of the burden of foodborne disease in 2010. PLoS Med 12 (12): e1001923. DOI: 10.1371/journal.pmed.1001923.

Hwang J, Park J. 2015. Characteristics of enterotoxin distribution, hemolysis, lecithinase, and starch hydrolysis of *Bacillus cereus* isolated from infant formulas and ready-to-eat foods. J Dairy Sci 98 (3): 1652-60. DOI: 10.3168/jds.2014-9042.

Jansson L, Hedman J. 2019. Challenging the proposed causes of the PCR plateau phase. Biomol Detect Quantif 17: 100082. DOI: 10.1016/j.bdq.2019.100082.

Jung J, Jin H, Seo S, Jeong M, Kim B, Ryu K, Oh K. 2022. Short communication: Enterotoxin genes and antibiotic susceptibility of *Bacillus cereus* isolated from garlic chives and agricultural environment. Intl J Environ Res Public Health 19 (19): 10-16. DOI: 10.3390/ijerph191912159.

Kavanaugh DW, Porrini C, Dervyn R. 2022. The pathogenic biomarker alcohol dehydrogenase protein is involved in *Bacillus cereus* virulence and survival against host innate defence. PLoS One 17: e0259386. DOI: 10.1371/journal.pone.0259386.

Kim CW, Cho SH, Kang SH, Park YB, Yoon MH, Lee JB, No WS, Kim JB. 2015. Prevalence, genetic diversity, and antibiotic resistance of *Bacillus cereus* isolated from Korean fermented soybean products. J Food Sci 80 (1): M123-M128. DOI: 10.1111/1750-3841.12720.

Lee H, Yoon Y. 2021. Etiological agents implicated in foodborne illness world wide. Food Sci Anim Resour 41 (1): 1-7. DOI: 10.5851/KOSFA.2020.E75.

López AC, De Ortuzar RVM, Alippi AM. 2008. Tetracycline and oxytetracycline resistance determinants detected in *Bacillus cereus* strains isolated from honey samples. Rev Argent Microbiol 40 (4): 231-236.

Lynn TM, Vijayalakshmi G, Vanajakshi V. 2014. Molecular characterization of enterotoxin genes from food-borne pathogen, *Bacillus cereus*. J Sci Innov Res 2 (1): 30-35.

Merzougui S, Lkhider M, Grosset N, Gautier M, Cohen N. 2014. Prevalence, PFGE typing, and antibiotic resistance of *Bacillus cereus* group isolated from food in Morocco. Foodborne Pathog Dis 11 (2): 145-149. DOI: 10.1089/fpd.2013.1615.

Messelhäusser U, Frenzel E, Blochinger C, Zucker R, Kampf P, Ehling-Schulz M. 2014. Emetic *Bacillus cereus* are more volatile than thought: Recent foodborne outbreaks and prevalence studies in bavaria (2007-2013). Biomed Res Int 2014: 465603. DOI: 10.1155/2014/465603.

Miao X, Zhu L, Bai X. 2022. Bacterial community assembly and beta-lactamase (bla) genes regulation in a full-scale chloraminated drinking water supply system. J Environ Chem Eng 10 (3): 107677. DOI: 10.1016/j.jece.2022.107677.

Nanteza H, Dewanti-Hariyadi R, Nurjanah S. 2022a. The occurrence of *Bacillus cereus* in white pepper from Bogor, Indonesia. IOP Conf Ser Earth Environ Sci 1097 (1): 012030. DOI: 10.1088/1755-1315/1097/1/012030.

Nanteza H, Dewanti-Hariyadi R, Nurjanah S. 2022b. Phylogenic identification of toxigenic *Bacillus cereus* in chilli and white pepper from Bogor area, Indonesia. An Univ "Dunarea de Jos" Galati Fasc VI Food Technol 46: 9-21. DOI: 10.35219/foodtechnology.2022.2.01.

Oktari A, Supriatin Y, Kamal M, Syafrullah H. 2017. The bacterial endospore stain on Schaeffer Fulton using variation of methylene blue solution. J Phys Conf Ser 812 (1): 012066. DOI: 10.1088/1742-6596/812/1/012066.

Paray AA, Singh M, Amin Mir M. 2023. Gram staining: A brief review. Intl J Res Rev 10 (9): 336-341. DOI: 10.52403/ijrr.20230934.

Rizki WM, Dewanti-Hariyadi R, Dewantari H. 2022. Comparison of predictive growth models for *Bacillus cereus* in cooked and fried rice during storage. An Univ "Dunarea de Jos" Galati Fasc VI Food Technol 46 (2): 89-103. DOI: 10.35219/foodtechnology.2022.2.07.

Roberts MC. 1996. Tetracycline resistance determinants: Mechanisms of action, regulation of expression, genetic mobility, and distribution. FEMS Microbiol Rev 19 (1): 1-24. DOI: 10.1111/j.1574-6976.1996.tb00251.x

Rodrigo D, Rosell CM, Martinez A. 2021. Risk of *Bacillus cereus* in relation to rice and derivatives. Foods 10 (2): 302. DOI: 10.3390/foods10020302.

Saeed BMS, Abbas BA, Al-Jadaan SAN. 2018. Molecular detection of tetracycline resistance genes. Basrah J Vet Res 17 (3): 223-234.

Shariati A, Maniya A, Mohammad AK, Abedinzadeh M, Ganjalishahi M, Maleki A, Heidary M, Khoshnood Saeed. 2022. The resistance mechanisms of bacteria against ciprofloxacin and new approaches for enhancing the efficacy of this antibiotic. Front Pub Health 10: 1025633. DOI: 10.3389/fpubh.2022.1025633.

Sik Z, Akan M. 2024. Determination of antibiotic resistance in *Salmonella typhimurium* and *Salmonella* kentucky serotypes of animal origin using conventional and molecular methods. Turkish J Vet Anim Sci 48 (1): 72-81. DOI: 10.55730/1300-0128.4338.

Skockova A, Cupakova S, Karpiskova R, Janstova B. 2012. Detection of tetracycline resistance genes in *Escherichia coli* from raw cow's milk. J Microbiol Biotechnol Food Sci 1: 777-784.

Son KL, Nugroho ASD, Rahayujati B, Gozali LK. 2020. Food poisoning outbreak caused by diarrhoeal *Bacillus cereus* in Tegalkenongo Village, Bantul, Yogyakarta, Indonesia: A retrospective study. Asia Pac Fam Med 18 (1): 1-5. DOI: 10.22146/APFM.V18I1.62.

Vardanyan R, Hruby V. 2006. Synthesis of Essential Drugs. Elsevier, Tucson, USA. DOI: 10.1016/B978-044452166-8/50036-4.

Zarei-Baygi A, Smith AL. 2021. Intracellular versus extracellular antibiotic resistance genes in the environment: Prevalence, horizontal transfer, and mitigation strategies. Bioresour Technol 319: 124181. DOI: 10.1016/j.biortech.2020.124181.

Zhuang M, Achmon Y, Cao Y, Liang X, Chen L, Wang, H, Siame BA, Leung KY. 2021. Distribution of antibiotic resistance genes in the environment. Environ Pollut 285: 117402. DOI: 10.1016/j.envpol.2021.117402.