

Beta hemolytic and beta-lactam antibiotic-resistant strain of *Lysinibacillus sphaericus* isolated from a tea growing region of West Bengal, India

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Manuscript received: 2 December 2024. Revision accepted: 10 February 2025.

Abstract. Nandi S, Bhattacharya M. 2025. Beta hemolytic and beta-lactam antibiotic-resistant strain of *Lysinibacillus sphaericus* isolated from a tea growing region of West Bengal, India. *Asian J Trop Biotechnol* 22: 1-6. River water and soil are primary sources of bacteria. Some of them often acquire pathogenicity under stressful environmental conditions. Regarding the pathogenicity of a bacterium, hemolysin is the most virulence factor. In this study, 16 isolates possess beta-hemolytic abilities out of 32 isolated copper tolerant bacteria. One of the strains was identified as *Lysinibacillus sphaericus* (NJCT1) using 16S rRNA sequencing. *Lysinibacillus sphaericus* is rarely pathogenic in humans but is known for its larvicidal and antagonistic activity in bacteremia-associated infections in immunocompromised patients. Kaljani River basin, the lifeline for the rapidly growing population in the tea-producing region of West Bengal, India, is the source of Gram-positive bacteria used in this study. Due to its pathogenic character, it can create a health problem for the locals if they use the river water frequently. The antibiotic susceptibility study, which involved testing the bacterial strain against a panel of commonly used antibiotics, showed that *L. sphaericus* (NJCT1) is resistant to 7 antibiotics (colistin, penicillin-g, amoxicillin, ceftazidime, cefuroxime, rifampin, and methicillin). This bacterial strain exhibited resistance against the β -lactam drug class, whereas complete susceptibility was recorded against drug classes like fluoroquinolones. As the most sensitive drug, fluoroquinolones could solve the problem caused by *L. sphaericus* isolated from the Kaljani River.

Keywords: Antibiotic sensitivity, bacteremia, *Lysinibacillus sphaericus*, pathogenicity, tea garden

INTRODUCTION

Under stressful conditions, microorganisms might become pathogenic. Bacteria often secrete extracellular proteins like coagulases, cytolytins, and enterotoxins to minimize competition, harm their adjacent cells, and exploit the nutrients (Tomita and Kamino 1997). Hemolysins have received the most attention among these toxins because their activity could be easily detected in mammalian erythrocytes. Red Blood Cells (RBC) could be lysed in the presence of hemolysins. Hemolysins can destroy cellular membranes, damage nearby tissues, cause cell lysis, and thus can be regarded as a crucial determinant of virulence in infection models of laboratory animals (Bhakdi et al. 1994; Tomita and Kamio 1997).

Alpha (α), beta (β), and gamma (γ) are three distinct types of hemolysin enzymes produced by bacteria. In some studies, distinctiveness between α and β hemolysis is essential to evaluate probiotics safety (Jeon et al. 2017). Still, other phenotypes are not crucial as they only indicate the virulence potential of those phenotypes (Ramachandran 2013). Bacterial isolates from environmental samples have rarely been tested for hemolysins (Albarral et al. 2016). However, in medical sectors, beta hemolysis is one of the important phenotypic features for identifying any bacteremia or septicemia-causing pathogenic bacteria, especially for group A and group B *Streptococcus* spp. (Nizet 2022).

Viable bacteria inside the blood are called 'bacteremia'. Bacteremia could originate from daily activities like oral hygiene or after undergoing some medical procedures. Asymptomatic bacteremia can enter the human bloodstream and lead to infections with symptoms of fever, hypotension, and chills. This disease can be life-threatening for those suffering from immune system disorders (Smith and Nehring 2017). Bacteremia is now one of the most common causes of death in non-coronary Intensive Care Units (ICU). It could be treated using antibiotics for patients with such infections. People with organ transplants, last-stage renal disease, diabetics, rheumatoid arthritis, and chemotherapy receivers are more likely to develop bacteremia-associated deadly infections, and antibiotics are effective in alleviating these infections. However, the efficacy of antibiotics seems to be in jeopardy since many bacteria have developed partial or total resistance against them. Excessive and misuse of antibiotics are the causes of bacterial resistance. Research policies and development efforts are required to find an effective solution to this problem of bacterial resistance (Larsson and Flach 2022).

River water is a significant reservoir of pathogenic bacteria. Previous studies have been done mainly on the enteric bacteria. Investigations on enteric bacteria are focused on their prevalence and antimicrobial assessment (Langendorf et al. 2015; Getie et al. 2019; Denku et al. 2022). Previous reports showed that patients who had undergone splenectomy, long-term intravenous drug users,

and people living with HIV commonly reported coping with bacteremia-based issues caused by these bacteria (Wenzler et al. 2015; Kinoshita et al. 2023; Meng et al. 2023). Rivers and its tributaries serve as water sources for the inhabitants of tea garden-dominated districts in the northern part of West Bengal. The river's water is used for drinking, other domestic purposes, and irrigation. The river also is a source of livelihood for fishermen living on both sides of the river. Several deaths have been reported in this region due to diseases caused by poor water quality (Sarkar 2012). Not only that, bacteremia-related diseases are now becoming a headache for the people living in this area. Therefore, the current study is critical, as people living in tea gardens adjoining the Kaljani River basin may unknowingly interact with beta-hemolytic bacteria regularly. Immuno-compromised individuals and people with external dermal wounds would be more susceptible to beta-hemolytic bacteria, resulting in bacteremia-associated disorders. Hence, the present investigation highlights the antibiotic susceptibility and resistance attributes of a beta-hemolytic strain, *Lysinibacillus sphaericus* (NJCT1), persisting in the Kaljani River basin.

MATERIALS AND METHODS

Study area and sample collection

Soil samples were collected from 14 tea gardens and 14 river beds near West Bengal, India's Indo-Bhutan border, covering Jalpaiguri, Alipurduar, and Kalimpong Districts. All collections were carried out from February 2022 to September 2023 in the early morning. In this research, the studied strain was isolated from the Kaljani River basin ($26^{\circ}34'30''\text{N}$ & $89^{\circ}25'29''\text{E}$ to $26^{\circ}34'27''\text{N}$ & $89^{\circ}25'22''\text{E}$), located in the Kalchini Sub-division under Alipurduar District of West Bengal, India. Nimtijhora tea garden is

situated in the Kaljani River basin and is one of the primary water sources for tea garden workers (Figure 1). This collection was done in the early morning of September 2022. Top soil samples were collected in a zigzag pattern, and about 10 samples were collected from this site and the distance of each sampling was about 100 meters. The collections were kept in sterile vials and instantly preserved inside an ice box during transportation to the laboratory, ensuring their integrity. The samples were mixed uniformly before laboratory experiments.

Isolation of pure bacteria

500 mg of each sample was taken into a sterile eppendorf containing 1 mL of distilled water to make soil suspensions. 500 μL of suspension was inoculated into 20 mL of nutrient broth, followed by 24 hours of incubation at 35°C (Saha et al. 2021). As the collection area is contaminated with dolomite debris and mining effluents, and copper is one of the predominant metals present in dolomite, the primary goal was to isolate copper-tolerant bacteria. The copper-tolerant bacteria were isolated by spread plate method on nutrient agar plates with different copper salt solutions ranging between 0.05 and 0.20 mg/mL and then incubated at 35°C . After 24 hours of incubation, distinct colonies were isolated and were streaked repeatedly to get the pure isolates. Morphological characters were done using a microscope.

Pathogenicity test

All the obtained isolates were assessed for pathogenicity through a blood agar test. 5% goat blood was added to 50% cooled blood agar base media, and the plates were prepared carefully without bubble formation. Isolates were individually streaked on the plates, followed by twenty-four hours of incubation at 35°C (Rosa-Fraile and Spellerberg 2017).

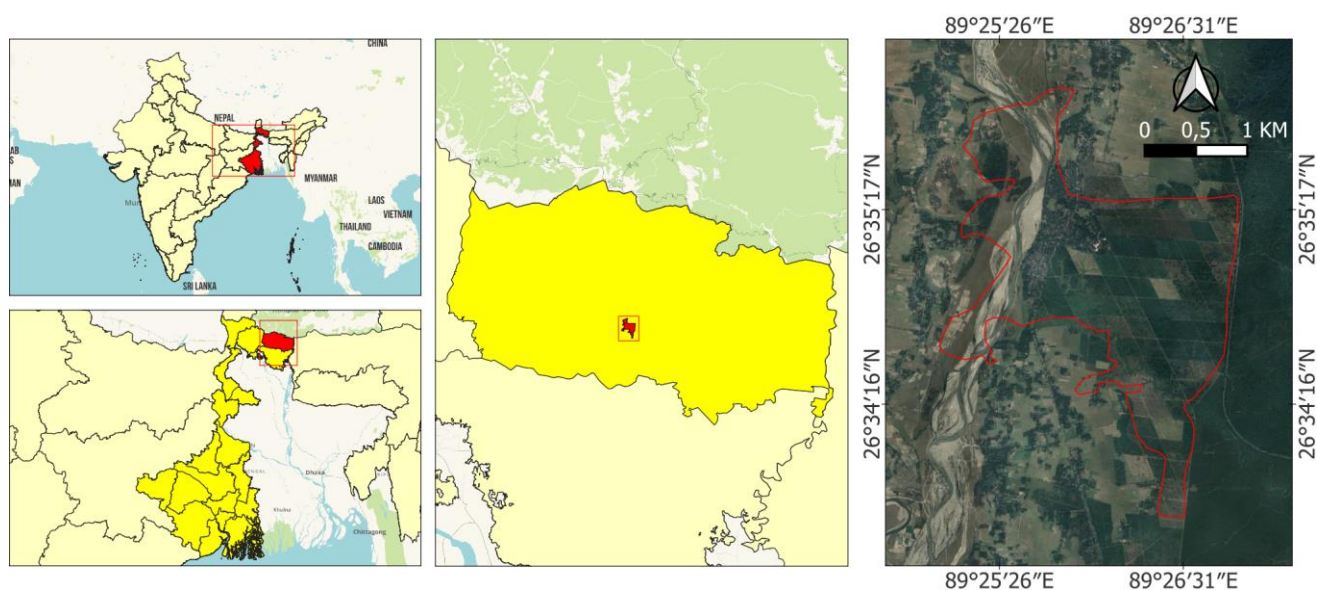


Figure 1. Map of the study area in Nimtijhora Tea Garden, Alipurduar, West Bengal, India

Molecular characterization

The isolated beta-hemolytic strain was characterized by 16S rRNA gene sequencing. For this purpose, the 16S rDNA region was isolated from 24-hour-old cultures. The isolated region of the studied strain was further amplified and purified through column purification. The purified product sequence was analyzed using Snager's method by Edison Life Science (India). The sequencing data result was then compared using BLAST analysis against the available sequence data in the NCBI database.

Antibiotic susceptibility test of studied strain

Antibiotic susceptibility profiling against selected strains was done using the Kirby Bauer disc diffusion procedure by Robert et al. (2009), according to the National Committee for Clinical Laboratory Standards (NCCLS). Then, 24 hours of the culture of strain was spread on Muller Hinton agar media using a sterile spreader. Natural, semi-synthetic, and synthetic antibiotics were selected to assess the sensitivity of the bacterial strain. Natural antibiotics include streptomycin, bacitracin, penicillin-g, colistin, pristinamycin, vancomycin, kanamycin, polymixin-b, spectinomycin; semi-synthetic antibiotics include amoxicillin, ceftazidime, cefoxitin, cefuroxime, imipenem, and azithromycin. synthetic antibiotics include teicoplanin, nitrofurantoin, tetracycline, tigecycline, rifampin, methicillin, linezolid, erythromycin, ofloxacin, fusidic acid, gemifloxacin, ciprofloxacin, enoxacin, co-trimoxazole, fosfomicin, clindamycin, gentamycin and novobiocin. The diameter of inhibition zones was measured in 'mm' and tabulated. They were further classified as susceptible, resistant, and intermediate towards antibiotics to determine their sensitivity.

RESULTS AND DISCUSSION

Bacteria isolation

After 24 hours of incubation on nutrient agar plates containing different ranges of copper salt concentrations, distinct colonies were observed on the plates made with 0.20 mg/mL copper salts. About 32 pure isolates with various morphological characters were obtained. Gram staining analysis showed that 28 isolates were Gram-positive bacilli and 4 were Gram-negative cocci.

Pathogenicity test

A blood agar test was conducted to determine the hemolytic capabilities of the isolates. A clear halo zone around the streaked lines of isolates depicted beta hemolysis (Figure 2). Green or brown color formation around the streaked line in the media depicted alpha hemolysis, whereas no halo zone or color formation confirmed gamma hemolysis. After 24 hours of incubation, 16 isolates showed clear zones around the streaked lines and exhibited beta-hemolytic capabilities, whereas the rest were gamma-hemolytic.

Molecular characterization

Through 16S rDNA identification, analyzed sequenced data showed a 1204 base pair and 99.24% homology with *L. sphaericus*, thus confirming the identity of one of the isolated beta-hemolytic strains. Further, the obtained sequence data was deposited in GenBank, and an accession number OP810683 was procured (Nandi et al. 2024). The previous case studies related to *L. sphaericus* showed that this isolate is a potential threat.

Antibiotic susceptibility test of studied strain

Among all the beta-hemolytic strains, *L. sphaericus* (NJCT1) was selected to evaluate its antibiotic susceptibility profiling. In this study, the antibiotic sensitivity of *L. sphaericus* (NJCT1) was assessed against 33 standard antibiotics (Figure 3; Table 1).



Figure 2. Clear halo zone around streak line of studied strain (NJCT1) on blood agar plate depicting beta hemolysis

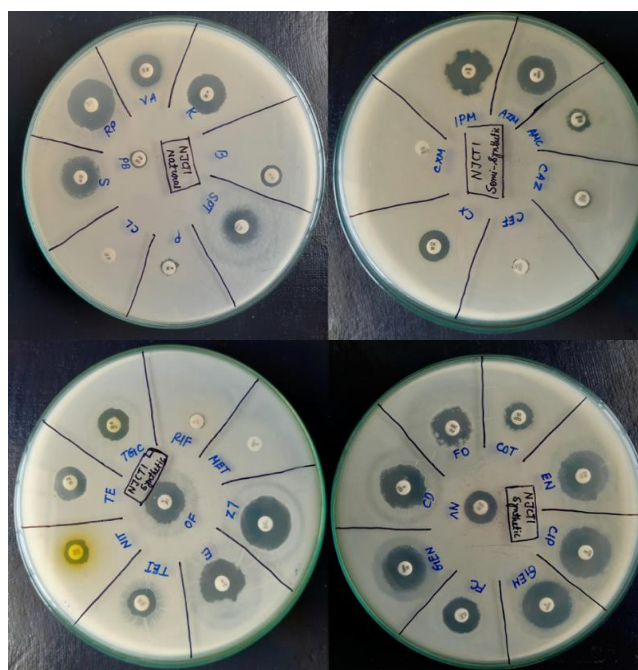


Figure 3. Antibiotic susceptibility of *Lysinibacillus sphaericus* (NJCT1) against several antibiotics

Table 1. Antibiotic susceptibility profile of *Lysinibacillus sphaericus* (NJCT1)

Drug class	Antibiotic	S/R/I
β lactam/ cephalosporin	Penicillin- G	R
	Methicillin	R
	Amoxicillin	R
	Cefuroxime	R
	Ceftazidime	R
Sulfonamide	Cefoxitin	S
	Co-trimoxazole	S
Fluoroquinolones	Ofloxacin	S
	Ciprofloxacin	S
	Enoxacin	S
	Gemifloxacin	S
Aminocoumarin	Novobiocin	S
Anti-mycobacterial	Rifampin	R
Aminoglycoside	Gentamycin	S
Glycylcycline	Tigecycline	S
Macrolide	Erythromycin	S
	Azithromycin	S
	Pristinamycin	S
Fosfomycin	Fosfomycin	S
Glycopeptide	Teicoplanin	S
	Vancomycin	S
Lincomycin	Clindamycin	S
Tetracycline	Tetracycline	S
Oxazolidinones	Linezolid	S
Nitrofurantoin	Nitrofurantoin	S
Fusidane	Fusidic acid	S
Polypeptide	Polymixin-B	I
	Colistin (Polymixin-E)	R
	Bacitracin	I
Carbapenems	Imipenem	S
Aminoglycosides	Streptomycin	S
	Spectinomycin	S
	Kanamycin	S

Note: 0.0-4.9 mm = Resistant (R); 5.0-9.9 mm = Intermediate (I); 10mm <= Susceptible (S)

Based on the National Committee for Clinical Laboratory Standards (NCCLS), the results showed *L. sphaericus* (NJCT1) had complete resistance against 7 antibiotics, namely 2 natural (colistin, penicillin-g); 3 semi-synthetic (amoxicillin, ceftazidime and cefuroxime); and 2 synthetic antibiotics (rifampin and methicillin). Based on the inhibition, *L. sphaericus* (NJCT1) showed intermediate resistance against 2 natural antibiotics (bacitracin and polymixin-b), and the rest were susceptible. Ciprofloxacin, gemifloxacin, ofloxacin, and enoxacin, belonging to the fluoroquinolones class, showed a 21 mm inhibition zone, the most sensitive antibiotic for *L. sphaericus*.

Discussion

In this study, 32 copper-tolerant bacteria have been isolated from river beds and tea gardens near the Indo-Bhutan border of West Bengal, India. Several bacteria possess beta-hemolytic abilities. Bacteria mainly possess three different types of hemolytic activities. Beta (β) hemolysis is indicated by total lysis of red blood cells, and hence, it is called "true lysis," visible as a clear and transparent area in blood agar culture. Alpha (α) hemolysis caused the increase of methemoglobin, indicated by a

brown or green area in the blood agar culture, suggesting that there is no lysis of hemoglobin but just a reduction of hemoglobin to methemoglobin. Gamma (γ) hemolysis or non-hemolysis indicates no cell damage or transformation in the agar plates. One of the isolates, *L. sphaericus*, with beta-hemolytic activity, was found in the Kaljani River basin. This isolate, which exhibits the highest activity, is an antagonist in rare cases of infection caused by bacteremia in patients with weakened immune systems. However, it is rarely pathogenic in humans. These findings could have significant implications for understanding and potentially treating such infections.

Lysinibacillus sphaericus (NJCT1), a Gram-positive bacillus spore-forming bacteria, might produce cytolytic toxin, a common toxin produced by Gram-positive bacteria (Masignani et al. 2006; Ramachandran 2013). Under extreme environmental conditions, *Bacillus* spp. also produces some toxins as a survival strategy. Bacteria mainly produce cytotoxin to access nutrients or eliminate competition from their surroundings (Harding et al. 2011). *L. sphaericus* was previously called *Bacillus sphaericus*, which is known for its insecticidal activity and hemolysin encoding genes, which include *hylA*, *hylC*, *hylD*, and *xhIA* are the crucial factors for this activity (Gómez-Garzón et al. 2017). *Bacillus* is not known for its pathogenicity. However, some *Bacillus*, such as *L. sphaericus*, could be an opportunistic pathogen due to its ubiquity and ability to cause severe human infections. The most commonly observed systematic infection due to *Bacillus* spp. is "bacteremia", which is generally associated with intravascular catheterization. Neutropenic patients, neonates, and intravenous drug users suffer the most from disseminated *Bacillus* spp infection. Various antibiotics were prescribed to treat *L. sphaericus*, as the obtained bacteria mixed with different blood cultures from different patients revealed susceptibility to various antibiotics (Wenzler et al. 2015).

Bacteremia can be life-threatening to immunocompromised people and is now a common problem with people dealing with some serious health issues. The water from the Kaljani River supports the livelihood of people and animals residing in the tea gardens. Infection of *L. sphaericus* originating from the Kaljani River could be dangerous. A detailed antibiotic study was conducted to understand the potential treatment of infection. The findings showed that *L. sphaericus* in this study is resistant to 5 out of 6 antibiotics belonging to the β-lactam drug class.

β-lactams (penicillin, cephalosporins, and monobactams) have been at the top of the antibiotic market since the beginning due to their efficiency against bacterial infections linked to other species that look like losing their edge, as bacteria have seemingly grown resistance against them in recent days. β-lactamase is the enzyme secreted by bacteria to attack the ring of β-lactam antibiotic, thus aiding in developing resistance in bacteria against this drug class (Pandey and Cascella 2019). Complete resistance was obtained against polymixin-e (colistin), and polymixin-b has an intermediate resistance, which can be a concern to clinical approaches as nowadays polymixins are regarded

as a gateway in multidrug resistance conditions (Poirel et al. 2017). Complete resistance of rifampin expressed that it might not inhibit the DNA-dependent RNA polymerase of the isolate, which generally aids in bactericidal activity (Campbell et al. 2001).

Resistance capability in bacteria is often associated with an isolate's heavy metal resistance pattern, as several studies stated the existence of both heavy metal and Antibiotic Resistance Genes (ARGs) in a bacterium, specifically with the occurrence of genes imparting heavy metal tolerance in the chromosome and antibiotic resistance in the plasmid. Further, the co-existence of heavy metal and antibiotic tolerance genes in plasmid conferring resistance to both stress factors has also been reported (Murray et al. 2024). A plasmid harboring heavy metal tolerance genes is more likely to possess ARGs than plasmids lacking heavy metal resistance genes. In this regard, *L. sphaericus* isolated from the Kaljani River grew at 0.20 mg/mL copper stress, illustrating its copper resistance capability. Wu et al. (2022) stated that isolates capable of tolerating copper up to 0.12 mg/mL are considered resistant and have copper detoxifying abilities. The resistance potential of *L. sphaericus* may contribute to its various antibiotics. Li et al. (2017) reported that resistance genes against multiple antibiotics, i.e., beta-lactam and polymyxin, are associated with heavy metal tolerant genes.

The result showed that fluoroquinolones were the most susceptible against *L. sphaericus* (NJCT1). This outcome is identical to the well-known occurrence of 12 *L. sphaericus* bacteremia infection cases in a Children's Cancer Hospital in Italy, reported in 10 years. Verification of 12 out of 469 cases (around 2%) of bacteremia infections, who were also cancer patients or were in the process of bone-marrow transplant were successfully treated with ciprofloxacin, which belongs to the fluoroquinolone drug class (Castagnola et al. 2001). This 2% success rate is significant as it demonstrates the potential of ciprofloxacin in treating *L. sphaericus* infections. Amoxicillin was recommended and had positive outcomes for two other immunosuppressed patients suffering from acute sepsis and dialysis-associated peritonitis (Wenzler et al. 2015; Kinoshita et al. 2023). However, *L. sphaericus* (NJCT1) in this study showed unusual in-vitro resistance against amoxicillin. Meng et al. (2023) reported that an HIV patient infected by the *L. sphaericus* (FY22) strain was treated with levofloxacin and vancomycin had an effective result. This study also observed the susceptibility of *L. sphaericus* (NJCT1) towards vancomycin.

In conclusion, bacteremia-associated infections are becoming a threat nowadays. Even though some significant infections associated with *L. sphaericus* have been reported, a detailed antibiotic study is essential to reduce the bacterial pool and, consequently, infections. Evaluating potential threats is greatly assisted by determining pathogenic phenotypes of environmental strains, identifying potential adaptations, and initiating clinically relevant bacteria. Antibiotic assessment of the *L. sphaericus* strain isolated from the Kaljani River basin can be a future

approach to control the possible threat posed by this species through its beta-hemolytic abilities.

ACKNOWLEDGEMENTS

Sudeshna Nandi would like to acknowledge the University Grant Commission (UGC), Govt of India, for providing the SJSJC fellowship (2022-23-UGCES-22-WESF-SJSJC-2360)

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