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Isolation and Characterization of Haemolytic *Bacillus cereus* from Black Rats

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Abstract

Bacillus cereus is a Gram-positive spore-forming bacteria belonging to *B. cereus* group in the genus *Bacillus*. The organism was reported as major food poisoning pathogen and responsible to cause diarrhoea and septicaemia. *B. cereus* was ubiquitous and frequently isolated from soil and environmental surfaces. Animals and insects were also reported to carry the pathogen. However, *B. cereus* was not isolated from rodents until now. In this study, we have isolated *B. cereus* from black rats (*Rattua rattus*) for the first time. A total of 13 (20.31%) out of 64 animals were positive for *B. cereus*. Sixteen isolates were recovered from both blood (4) and faecal (12) samples. All isolates were detected as *B. cereus* by VITECK 2 compact automated system. Gram staining revealed all isolates are Gram-positive rods and β-haemolysis was observed on blood agar. Molecular identification with 16S rRNA sequences revealed that all isolates have shared 99.67 to 99.78% similarity with *B. cereus*. In the phylogenetic tree, all isolates were formed as four groups and branched with *B. cereus* group. The study indicating that *B. cereus* may be a new zoonotic risk to humans associated with rodents.

Keywords

Gram-positive spore-forming bacteria, *Rattua rattus.*

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INTRODUCTION

Bacillus cereus is Gram-positive, aerobic spore-forming bacteria with clinical importance and causing a range of symptomatic diseases [1]. Taxonomically, the organism is belonging to *B cereus* group, which comprise 8 closely related species including *B. thuringiensis*, *B. cereus, B. toyonensis* and *B. anthracis*. *B cereus* was initially considered as non-pathogenic environmental organism, however, it was later identified as pathogenic species when it was identified from intestinal disease, and now it has been considered as important pathogen that cause non-intestinal and fatal diseases [2]. *B cereus* is responsible for food poisoning and causes diarrhoea. It has been considered as second most pathogenic species in food borne outbreaks in France and third most agent in Europe [3]. The vegetative cells of *B. cereus* were ingested as viable cells and secrets enterotoxins in the small intestine which induces food poisoning that in turn manifested as diarrhoea [4]. In addition to food poisoning, *B. cereus* was also responsible to induce local and systemic infections [1, 5]. The infections and mainly described clinical symptoms caused by the pathogen includes septicaemia, pneumonia, meningitis, encephalitis, endocarditis and endophthalmitis. In the immunocompromised patients, the infections become fatal and results in patient deaths up to 10% of cases [6, 7].

The organism is widely spread in natural environments and commonly isolated from soil, food and plants [4]. The organism was also isolated from insect larva and human skin [3] and found to be grown in the intestinal tract of mammals and insects [4]. However, no report was made to isolate *B. cereus* from rodents till date. Rodents are reported as potential to transmit number zoonotic infections to humans including bacterial, fungal, viral and protozoal and responsible for outbreaks of plagues. They also capable of hosting a diversified bacterial pathogens including *Bacillus* species [8]. However, as per our knowledge, *B. cereus* was not detected until now from rodent species. In this study, we reported the isolation and characterization of *B. cereus* bacteria from the blood and faecal samples of black rats *(Rattus rattus*) for the first time.

MATERIALS AND METHODS

Sample Collection

Rats were captured using locally available iron mesh made traps randomly from different locations in Nellore city (southern India). Collected animals were brought to laboratory at Department of Biotechnology, Vikrama Simhapuri University, Nellore, India and identified morphologically to gender and species level [9]. Animals were anesthetised by following standard procedure [10] and sacrificed by cervical dislocation. Animals were sacrificed as per the norms of animal ethical committee. Blood sample (1-2 ml) was collected from each animal aseptically by cardiac puncture into sterile EDTA coated tubes. A loopful of faecal sample was also collected from rectum of each animal.

Bacterial Isolation and Identification

Fresh blood samples were (100 µl) inoculated on sheep blood agar plates and incubated at 37°C for 24-48 hours. Collected faecal samples were inoculated into nutrient broth (NB) and incubated at 30°C aerobically. After 24 hours of incubation, the resultant NB growth was inoculated on blood agar plates and incubated under the same conditions. After the completion of incubation, suspected colonies (feathery, grey in colour and opaque with rough surface) were subculture from each plate onto new blood agar plates and incubated under the same conditions and observed for haemolysis. Gram-staining was done for all bacterial isolates for morphological identification. All the isolates were identified by biochemical detection with VITECK 2 Compact Automated System (BioMérieux, India) using BCL ID cards by following the manufacturer's instructions [11].

Molecular Detection

All the isolates were subjected to molecular screening for further identification. Bacterial isolates were inoculated into tryptic soya broth (TSB) and incubated overnight at 30°C [12]. Overnight grown cultures were processed for genomic DNA extraction by using QIAamp UCP Pathogen Mini Kit (QIAGEN, New Delhi, India) by following the manufacturer's instructions. The 16S rRNA gene was amplified from all extracted genomic DNA samples using universal primers P8 and Pc1544 [13]. Amplified DNA fragments were purified and sequenced with 3730xl DNA Analyzer (Applied Biosystems). The nucleotide sequences were searched against DNA sequences in BLAST (https://blast.ncbi.nlm.nih.gov) for homology and species identification. Phylogenetic tree was constructed with the related sequences available in DNA database (https://www.ncbi.nlm.nih.gov/) using Neighbour-Joining method in MEGA v10.2 with 1000 bootstrap replicates (www.megasoftware.net).

RESULTS

The present study was conducted as a part of investigation conducted for the prevalence of bacterial pathogens associated with rodents in Nellore city, India. We have collected a total of 64 rats and identified them as *Rattus rattus* (black rats). *B. cereus* colonies were recovered from 13 out of 64 animals (20.31%). Out of 64 blood and faecal samples, 4 blood samples and 12 faecal samples were found to be positive for *B. cereus*. Gram staining revealed morphology of isolates as Gram-positive rods under the microscopic observation. Based on biochemical identification by VITEK 2 automated system, all the isolates were identified as *Bacillus cereus* with 93 to 99 % probability. All isolates were shown positive for utilization of various amino acids. The results of biochemical analysis for isolates are given in Table 2. Beta-haemolysis was observed for all isolates on blood agar plates.

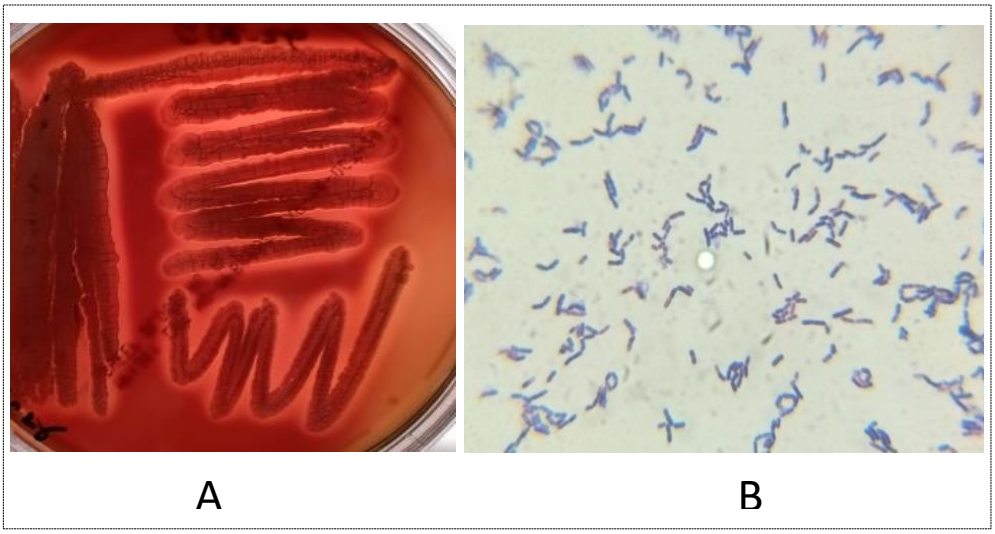

Figure 1. *Bacillus cereus* on blood agar showing β-haemolysis (A), Gram (+)ve rods of *B. cereus* were observed under microscope after Gram staining (B).

Table 1. Morphological characterization of isolates

Morphological feature	Bacterial Isolates			
	NLR A2	NLR A6	NLR A12	NLR A15
Cell morphology	Bacilli	Bacilli	Bacilli	Bacilli
Cell size	3.0-5.0 µm	3.0-5.0 µm	3.0-5.0 µm	3.0-5.0 µm
Gram-staining	Gram positive	Gram positive	Gram positive	Gram positive
Pigmentation	Pale Yellow	Pale Yellow	Pale Yellow	Yellow
pH	4.9-9.3	4.9-9.3	4.9-9.3	4.9-9.3
Haemolysis	β-haemolysis	β-haemolysis	β-haemolysis	β-haemolysis

Table 2. Biochemical characterization of all bacterial isolates recovered from rats.

S.No.	Biochemical Test	Bacterial Isolates			
		NLR A2	NLR A6	NLR A12	NLR A15
1.	BXYL	+	+	+	-
2.	LysA	-	-	-	-
3.	AspA	-	-	+	-
4.	LeuA	-	-	-	-
5.	PheA	+	+	-	+
6.	ProA	-	-	-	-
7.	BGAL	-	-	-	-
8.	PyrA	+	+	+	+
9.	AGAL	-	-	-	-
10.	AlaA	+	+	+	+
11.	TyrA	+	+	+	+
12.	BNAG	+	+	+	+
13.	APPA	-	-	-	-
14.	CDEX	-	-	-	-
15.	dGAL	-	-	-	-
16.	GLYG	-	-	-	-
17.	INO	-	-	-	-
18.	MdG	-	-	-	-
19.	ELLM	-	+	+	+
20.	MdX	-	-	-	-

21.	AMAN	-	-	-	-
22.	MTE	+	+	+	+
23.	GlyA	-	-	-	-
24.	dMAN	-	-	-	-
25.	dMNE	-	-	-	-
26.	dMLZ	-	-	-	-
27.	NAG	+	+	+	+
28.	PLE	-	-	-	-
29.	IRHA	-	-	-	-
30.	BGLU	+	+	+	+
31.	BMAN	-	-	-	-
32.	PHC	-	-	-	-
33.	PVATE	+	+	+	+
34.	AGLU	-	-	-	-
35.	dTAG				
36.	dTRE	+	+	+	+
37.	INU	-	-	-	-
38.	dGLU	+	-	+	+
39.	dRIB	-	+	+	+
40.	PSCNa	-	-	-	-
41.	NaCl	+	+	-	+
42.	KAN	+	+	+	+
43.	OLD	-	-	-	-
44.	ESC	+	+	+	+
45.	TTZ	-	-	-	-
46.	POLYB I	+	+	+	+

Abrivations: 1. BXYL: BETA-XYLOSIDASE, 2. Lysa: L-Lysin-ARYLAMIDASE, 3. AspA: L-Aspartate- ARYLAMIDASE, 4. LeuA: Leucine−ARYLAMIDASE, 5. PheA: PhenyLaLanine ARYLAMIDASE, 6. ProA L−ProLine ARYLAMIDASE, 7. BGA: BETA−GALACTOSIDASE, 8. PyrA: L−PyrroLydonyL−ARYLAMIDASE, 9. AGAL: ALPHA−GALACTOSIDASE, 10. AlaA: ALanine ARYLAMIDASE, 11. TyrA: Tyrosine ARYLAMIDASE, 12. BNAG: BETA−N−ACETYL−GLUCOSAMINIDASE, 13. APPA: ALa−Phe−Pro ARYLAMIDASE, 14. CDEX: CYCLODEXTRIN, 15. dGAL: D−GALACTOSE, 16. GLYG: GLYCOGEN, 17. INO: myo−INOSITOL, 18. MdG: METHYL−A−D−GLUCOPYRANOSIDE acidification, 19. ELLM: ELLMAN, 20. MdX: METHYL−D−XYLOSIDE, 21. AMAN: ALPHA−MANNOSIDASE, 22. MTE: MALTOTRIOSE, 23. GlyA: GLycine ARYLAMIDASE, 24. dMAN: D−MANNITOL, 25. dMNE: D−MANNOSE, 26. dMLZ: D−MELEZITOSE, 27. NAG: N−ACETYL−D−GLUCOSAMINE, 28. PLE: PALATINOSE, 29. IRHA: L−RHAMOSE, 30. BGLU: BETA−GLUCOSIDASE, 31. BMAN: BETA−MANNOSIDASE, 32. PHC: PHOSPHORYL CHOLINE, 33. PVATE: PYRUVATE, 34. AGLU: ALPHA−GLUCOSIDASE, 35. dTAG: D−TAGATOSE, 36. dTRE: D−TREHALOSE, 37. INU: INULIN, 38. dGLU: D−GLUCOSE, 39. dRIB: D−RIBOSE, 40. PSCNa: PUTRESCINE accimiLation, 41. NaCl: GROWTH IN 6.5% NaCl, 42. KAN: KANAMYCIN RESISTANCE, 43. OLD: OLEANDOMYCIN RESISTANCE, 44. ESC: ESCULIN hydrolysis, 45. TTZ: TETRAZOLIUM RED, 46. POLYB-R: POLYMIXIN_B RESISTANCE.

Molecular Identification and Phylogenetic Analysis

The 16S rDNA sequences of all isolates were submitted to BLAST search against the sequences available in database. Nucleotide sequences of all isolates were identified as *Bacillus cereus* upon BLAST search and shared 99.67 to 99.78% similarity with *B. cereus* strain CCM2010. Multiple sequence alignment was performed using Clustal W in MEGA software with nucleotide sequences of all isolates along with the reference sequences from NCBI database. One hundred and seventeen 16S rDNA sequences belonged to *Bacillus* species available in NCBI database were used for multiple sequence alignment and phylogenetic tree construction to identify the better taxonomic position of all isolates. Nucleotide sequences of all 117 *Bacillus* species were formed as 10 separate clades. In the phylogenetic tree, species of the *B. cereus* group including *B. anthracis, B. cereus, B. thuringiensis* and *B. albus* were formed as a separate clade (coloured in red) (Figure 2). The sequences of all recovered isolates were formed as four groups (NLR A2, NLAR A6, NLR A12 and NLR A15) in the constructed phylogenetic tree and branched with *B. cereus* CCM2010 in the

pathogenic clade. The 16S rDNA sequences in the group NLR A2 shared 99.69%, NLR A6 shared 99.72%, NLR A12 shared 99.67%, and NLR A15 shared 99.78% similarities with *B. cereus.*

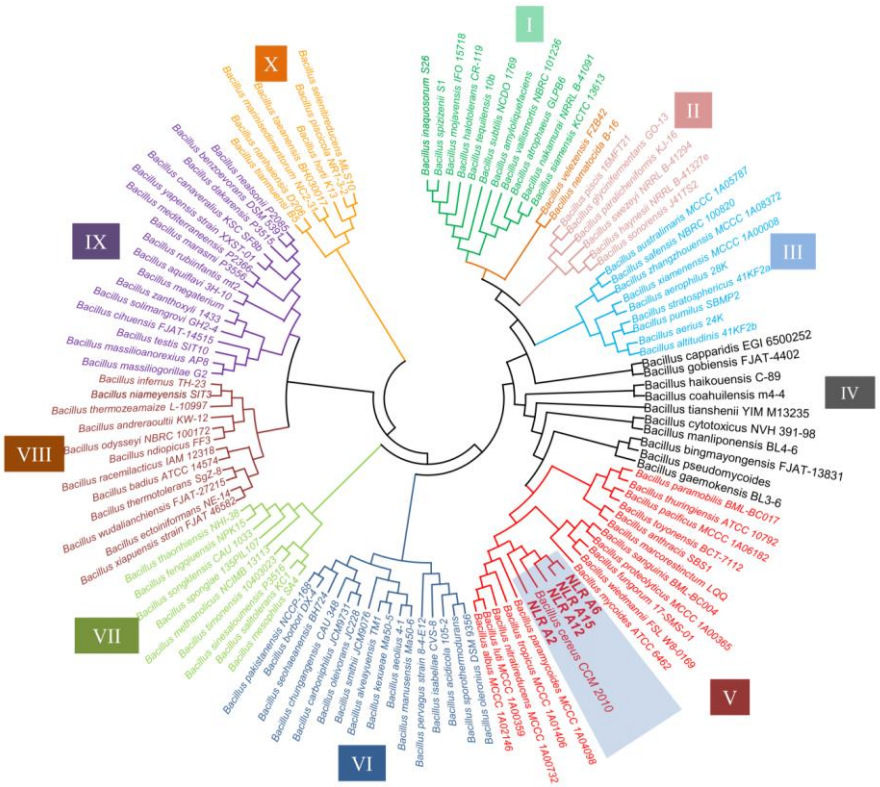

Figure 2. Phylogenetic tree of *Bacillus* species constructed based on 16S rRNA sequences. The evolutionary history was inferred using the Neighbour-Joining method with 1000 bootstrap replicates. All the recovered isolates were arranged into 4 groups and branched with *B. cereus*. All 117 *Bacillus* species are formed as total of 10 separate clades represented with different colours.

DISCUSSION

B. cereus is Gram-positive rod-shaped bacteria belonging to genus *Bacillus* of family Bacillaceae. The genus *Bacillus* comprising more than 260 described species, of which a group of spore-forming bacteria with potential pathogenic factors, comprised with at least 8 closely related species, named as *Bacillus cereus* group. The species of this group include *B. anthracis*, *B. mycoides*, *B. cereus*, *B. cytotoxicus*, *B. thuringiensis*, B. *pseudomycoides*, *B. weihenstephanensis* and *B. toyonensis.* Except *B. cytotoxicus,* the genome of the group is found to be highly conserved and the 16S rRNA sequences are very similar to each other [14, 15]. *B. cereus* is well known to associate with food poisoning, and a wide variety of serious infections related to central nervous system, cardiac and some eye infections. [6, 16]. *B. cereus* was isolated from air in a cow shed for the first time, however, it was identified as food poisoning organism in the mid of 20th century [17]. More recently, this organism was recognised as potential etiologic agent of localised and systemic infections [6].

B. cereus is ubiquitous and commonly found in soil and different environmental surfaces. It was also been isolated from intestines of mammals and insects [4]. The other species of the *B. cereus* group isolated from different samples including environmental and animals like *B. thuringiensis* from silkworm larvae, *B. anthracis* from cattle [14, 18]. However, no species from *B. cereus* group was isolated or reported from rodent species till date. In this study, we have isolated *B. cereus* from *Rattus rattus* from both blood and faecal samples. To our knowledge, this is the first report to isolate *B. cereus* from rodent species. Rodents hosts a wide verity of zoonotic pathogens, which can be transmitted to humans through direct contact with rats and indirectly by vector species. A wide range of pathogenic bacterial species are harboured by rodents, which are not causing any significant illness in animals. The bacterial species *Anaplasma*, *Bartonella*, *Leptospira*, *Borrelia*, *Coxiella*, *Francisella*, *Ehrlichia*, *Rickettsia* and *Yersinia* are majorly associated with rodents with potential pathogenicity [9]. Few studies were also reported on the isolation

of *Bacillus* species from rodents along with other bacterial species [19].

In this study, rats were captured for the investigation of bacterial pathogens associated with rodents and zoonotic risk of humans from rodents survived in Nellore municipality. A total of 16 isolates were recovered from rats. The 16S rRNA sequences of all isolates were highly similar to *B. cereus* by sharing 99.67 to 99.78% similarity and cladded with *B. cereus* group in the phylogenetic tree. The pathogen reported to cause diseases by producing enterotoxins. Haemolysin BL (HBL) and nonhemolytic enterotoxin (NHE) are the two major toxins secreted by *B. cereus*. Both are responsible for diarrhoea-type food poisoning [20]. Besides gastrointestinal infections, this pathogen was reported to cause serious fatal conditions including the infections related to central nervous system, respiratory and urinary tract infections, endophthalmitis, septicaemia and endocarditis [6]. Isolation of such potential pathogenic species *B. cereus* from rodents indicating that rodents can host new pathogens and risk of new rodent-borne zoonosis in future.

CONCLUSION

B. cereus is spore forming food poisoning and pathogenic bacteria. It has incriminated to cause other serious infections like central nerve infections, respiratory tract, and endocarditis. The organism was frequently isolated from soil and plants. This pathogen was also isolated from intestines of animals and insects. No report was made on the isolation of *B. cereus* from rodents. In this study, we have isolated *B. cereus* from black rats, indicating that this organism may be a new zoonotic risk of humans associated to rodents.

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CONFLICT OF INTEREST

No conflict of Interest was found between authors.

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