

VETERINARSKI ARHIV 85 (3), 347-357, 2015

***Bacillus cereus* harboring the pXO1 plasmid with *pag* gene causes anthrax-like fatal septicemia in immunosuppressed cattle - short communication**

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CHANDRANAİK, B. M., P. GIRIDHAR, H. K. MUNIYELLAPPA, R. HEGDE, N. EARANNA, D. RATHNAMMA, R. S. KALGE, S. KANAKA, G. K. CHANDRAKALA, ASHAMAYANNA, M. D. VENKATESHA: *Bacillus cereus* harboring the pXO1 plasmid with *pag* gene causes anthrax-like fatal septicemia in immunosuppressed cattle - short communication. Vet. arhiv 85, 347-357, 2015.

ABSTRACT

Bacillus cereus is ubiquitous in nature and while most isolates appear to be harmless, some are associated with food-borne illnesses, wound infections, endocarditis, osteomyelitis, endophthalmitis and urinary tract infections in humans. Recently, a few isolates have been identified as the causative agents of anthrax-like severe pneumonia in humans, and these isolates were found to harbor most of the *B. anthracis* virulence plasmid pXO1. Here we report the characterization of three clinical *B. cereus* isolates recovered from heart blood and spleen samples of cattle which had died with 'anthrax like' symptoms. Apart from the cultural characterizations, primers targeting the 16S rRNA gene of *B. cereus* were designed and used on these isolates. The isolates were found to harbor the pXO1 plasmid and lacked pXO2 plasmid. Further characterization of the pXO1 plasmid revealed that the isolates contained *pag*, *lef* and *cya* genes, which code for protective antigen, lethal factor and edema factor toxins responsible for eliciting an 'anthrax like disease' in cattle. The sequencing and phylogenetic analysis of partial *pag* gene sequences of *B. cereus* isolates were identical to *pag* gene sequences on the pXO1 of *B. anthracis*. In a pathogenicity test on mice, *B. cereus* isolates, when inoculated by the intra peritoneal route, caused mortality of the mice within 6 hours post inoculation.

Key words: *Bacillus cereus*, pXO1 plasmid, *Bacillus anthracis*, *pag* gene

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Introduction

The *Bacillus cereus* species complex is a set of ubiquitous, rod-shaped Gram-positive soil bacteria, with six subspecies that are genetically very similar but which have highly specialized lifestyles with distinct virulence spectra. The *B. cereus* species complex includes the opportunistic pathogen *B. cereus sensu stricto*, which is frequently implicated in cases of food poisoning, periodontitis and endophthalmitis, the entomopathogen *B. thuringiensis*, which is widely used as a bio-pesticide, and the causative agent of anthrax, *B. anthracis* (IVANOVA et al., 2003; WILSON et al., 2011). Their main distinguishing phenotypic features, including their respective virulence properties, are directly associated with large plasmids. In *B. anthracis*, the toxin and capsule genes responsible for anthrax disease are located on the 182-kb pXO1 and 95-kb pXO2 plasmids, respectively. Both plasmids also encode regulator genes that control the expression of anthrax virulence. In recent years, various clinical isolates of *B. cereus* group members have been found with large plasmids that are related to pXO1 and pXO2. Some of these plasmids are known to encode the genetic determinants that confer pathogenic properties (HU et al., 2009). *B. cereus* has been implicated in anthrax-like diseases, with the clinical presentation of severe pneumonia, affecting humans in North America (HOFFMASTER et al., 2004; HOFFMASTER et al., 2006; AVASHIA et al., 2007) and great apes in Africa (KLEE et al., 2010). Furthermore, *B. cereus* has been reported to cause fatal septicaemia in captive psittacines (GODOY et al., 2012). Most of these isolates possess the pXO1 plasmid encoding the anthrax toxin genes, and some are capable of producing the typical protective poly-D-glutamic acid capsule that shields the infecting bacterium from the immune system.

In this paper we report the isolation of three *B. cereus* isolates harboring the pXO1 plasmid, recovered from heart blood and spleen samples of immunosuppressed cattle that had died after suffering from foot and mouth disease, in an anthrax endemic region in Karnataka State, India. We also report the pathogenicity of these isolates in mice and the molecular characterization of these three *B. cereus* clinical isolates.

Materials and methods

Sample collections and origin of B. cereus isolates. Three isolates of *Bacillus cereus* described in this study were isolated from heart blood and spleen samples collected during the postmortem of cattle that had died after suffering from foot and mouth disease. The samples were collected at three sites, namely: Agrahara, Seemangala and Mulabagilu in the Kolar district of Karnataka State, which is a designated endemic district for anthrax in the state. The isolations were made as per standard microbiological protocols (HOFFMASTER et al., 2006; WILSON et al., 2011). *Bacillus cereus* isolated from the soil was used as an environmental isolate and the *B. anthracis* isolate used for comparison was

provided by the anthrax vaccine production center of the Institute of Animal Health and Veterinary Biologias, Bangalore, Karnataka, India.

Biochemical and phenotypic characterization. The *B. cereus* isolates were characterized by standard microbiological methods (LOGAN and TURNBULL, 1999; TOURASSE and KOLSTO, 2008). Motility was determined by microscopic observation of wet mounts of cells, grown in soybean casein digest broth. The test to determine susceptibility to gamma phage was performed as per the procedures of BROWN and CHERRY (1955). Capsule staining of cells was performed on cells incubated at 30 °C and 37 °C in heart infusion broth, supplemented with 0.8 % sodium bicarbonate and 50 % horse serum. Capsules were visualized using India ink, as described previously (HOFFMASTER et al., 2004).

Mice pathogenicity test. All mouse experiments were conducted in accordance with the institutional animal ethics committee regulations. Six-week-old, inbred, Swiss albino mice were housed in cages and provided food and water *ad libitum*. Spores for the mouse challenge experiments were produced as described by DE et al. (2002). Spores were diluted to a dose of 1.1×10^4 to 2.7×10^4 colony-forming units in sterile water and inoculated into the mice by the intra peritoneal route, as described by HOFFMASTER et al. (2004). A total of six mice were used for testing the pathogenicity of each isolate.

DNA extraction and purification. Genomic DNA and plasmid DNA were isolated from the disrupted cells using extraction kits (Amnion Biotech Pvt. Ltd. Bangalore), following the protocol provided by the manufacturer.

Polymerase chain reaction. 16S rRNA gene based PCR. We designed a set of primers: Forward primer 5' CWG RCC TAN CAC ATG SAA GTC 3' and Reverse primer 5' GRC GGW GTG TAC NAG GC 3' for amplification of the 16S rRNA gene of *B. cereus*. The reaction mixture included 50 ng of DNA, 100 ng of Forward Primer, 100 ng of Reverse Primer, 2 µL of 10 mM dNTPs mix, 5 µL of 10X *Taq* Pol. Buffer, 3 U of *Taq* Polymerase enzyme and PCR grade water to make the volume to 50 µL. The PCR included initial denaturation at 94 °C for 5 min, and 35 repeated cycles of denaturation at 94 °C for 30 sec, annealing at 55 °C for 30 sec, extension at 72 °C for 1.30 min and a final extension of 5 min at 72 °C. The PCR products were electrophoresed in 1 % agarose gel along with a 500 bp DNA ladder.

PCR amplification of pXO1 and pXO2 plasmids. The set of primers described by HOFFMASTER et al. (2006), which are known to amplify conserved sequences of pXO1 and pXO2 genes present in *B. anthracis* and pathogenic *B. cereus*, were used in this study. The primers for amplification of 512 bp sequences on pXO1 were: Forward primer (start nucleotide 2838 on pXO1) 5'CTAGAACTTACTGATACGGAGTG 3'; Reverse primer (start nucleotide 3348 on pXO1) 5'TTCAGTACCTTTTATCTACCCA 3'. The primers for amplification of 674 bp sequences on pXO2 plasmid amplification were: Forward

primer (start nucleotide 26513 on pXO2) 5' ACCACAAAAGGGCATCTT 3'; Reverse primer (start nucleotide 27186 on pXO2) 5' ACAGTAGTACTGGCAGGGTGT 3'. The PCR mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.001 % (wt/vol) gelatin, 0.2 mM of each dNTP, 20 pmol of each primer, 2.5 U of Taq DNA polymerase, and approximately 1 ng template DNA in a 100 µL total reaction volume. Template DNA was initially denatured by heating at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and primer extension at 72 °C for 1 min. Incubation for 5 min at 72 °C followed to complete the extension. DNA extracted from *B. anthracis* and environmental isolates of *B. cereus* were included in each set of PCR reactions. PCR amplicons were analyzed by electrophoresis through 2 % agarose gel.

PCR amplification of protective antigen gene (pag) on the pXO1 plasmid. Primer sequences and PCR protocols approved by WHO for amplification of the *pag* gene of *B. anthracis* were used for identifying whether the clinical isolates of *B. cereus* obtained during this study harbored the virulent *pag* gene on their pXO1 plasmid (INOUE et al., 2004). The details of the primer sequences used are depicted in Table 1.

PCR amplification of lethal factor (lef) and edema factor (cya) genes on the pXO1 plasmid. The primer sequences and PCR conditions described by OKINAKA et al. (1999) were used for identifying whether the clinical isolates of *B. cereus* obtained during this study harbored the toxin coding *lef* gene (coding for lethal factor toxin) and *cya* gene (coding for edema factor toxin) on their pXO1 plasmid. The details of the primer sequences used are depicted in Table 1.

PCR amplification of capsule (cap) gene on pXO2 plasmid. Primer sequences and PCR protocols approved by WHO for amplification of partial sequences on capsule (*cap*) gene of *B. anthracis* were used to determine whether the clinical isolates of *B. cereus* obtained during this study harbored the capsule gene on their pXO2 plasmid (INOUE et al., 2004). The details of the primer sequences used are depicted in Table 1.

Sequencing and phylogenetic analysis—The PCR amplified products were extracted from agarose gel and eluted in 25 µL nuclease free water using a Quiagen® gel extraction kit, and submitted for nucleotide sequencing at Amnion Biotech Pvt. Ltd, Bangalore, India. The nucleotide sequences were aligned with the sequences published in GenBank. A phylogenetic tree was constructed and sequence analysis was performed using MEGA version 6.2 software, by the maximum parsimony method with 500 bootstrap replicates (CHANDRANAİK et al., 2012).

Results

Sample collection. The state of Karnataka, India, witnessed one of the worst foot and mouth disease outbreaks in its history from June to October, 2013. The outbreaks caused

the deaths of more than six thousand cattle and the Kolar district of the state alone saw the death of more than two thousand cattle. The Kolar district is the highest milk producer in the state, with more than 85 % of its cattle being very high yielding cross breeds. The region is a designated anthrax endemic belt in the state, which records the highest number of anthrax outbreaks annually. During this epidemic of FMD, cattle died due to several reasons, of which the most important and the most common were secondary bacterial complications. The extensive vesicular lesions caused by FMD rendered the animals recumbent, starved, dehydrated, immunosuppressed, and this culminated in death. During the investigation of these outbreaks in three villages in the Kolar district, cattle showed 'anthrax like' symptoms of sudden death, heavy fluid accumulation in the thorax and abdomen, hemorrhages throughout the body and characteristic splenomegaly.

Phenotypic analysis. Heart blood and spleen samples collected during necropsy on these animals were subjected for culture, as per the standard microbiological protocols. Pure cultures of *B. cereus* were recovered from these samples. The *B. cereus* isolates were hemolytic, motile, and resistant to lysis by gamma-phage, and had phenotypes typical of the species. All three isolates produced similar colony morphologies of large, convex, smooth colonies and were negative for capsule by India ink staining. The *B. cereus* isolates obtained from Agrahara, Seemangala and Mulabagilu were named as *B. cereus* IAHV 2013 Agrahara isolate, *B. cereus* IAHV 2013 Seemangala isolate and *B. cereus* IAHV 2013 Mulabagilu isolate, respectively.

Polymerase chain reaction and phylogenetic analysis. Polymerase chain reaction (PCR), targeting the conserved region on the 16S rRNA gene of *B. cereus* yielded amplicons of about 1450 bp on the genomic DNA of all three clinical isolates. The PCR products were sequenced and phylogenetic analysis of the derived sequences revealed the highest sequence identity with other *B. cereus* isolates. However, 16S rRNA gene based PCR could not distinctly differentiate the *B. cereus* isolates from other pathogenic *Bacillus cereus* group members, *B. anthracis* and *B. thuringiensis*.

The PCR employed for detection of 512 bp length conserved sequences of the pXO1 plasmid yielded specific amplification in the clinical isolates of *B. cereus* and the positive control *B. anthracis*. The environmental isolate of *B. cereus* did not yield amplification.

The PCR employed for amplification of specific regions on the pXO1 plasmid, coding for protective antigen (*pag* gene), lethal factor (*lef* gene) and edema factor (*cya* gene), yielded specific amplicons demonstrating the presence of anthrax toxin genes in the *B. cereus* isolates obtained during this study (Table. 1).

The set of primers for amplification of the conserved genomic region of pXO2 plasmid and the genomic region on pXO2 coding for the capsule (*cap* gene) did not yield amplifications in the three clinical *B. cereus* isolates, nor in the environmental *B. cereus*

Table.1. Details of the primers used for *pag*, *lef*, *cya* genes on pXO1 and *cap* gene on pXO2 plasmids and PCR results

Gene Targeted	Forward (F) and Reverse (R) primer sequences (5' to 3')	Predicted Amplicon size (bp)	PCR Results				
			<i>B. cereus</i> Mulabagilu isolate	<i>B. cereus</i> Agraphara isolate	<i>B. cereus</i> Seemangala isolate	<i>B. cereus</i> Environmental isolate	<i>B. anthracis</i> (Positive control)
Protective antigen (<i>pag</i>) gene on pXO1 plasmid	F: TCCTAACACTAACGAAAGTCG R: GAGGTAGAAGGATATACGGT (INOUE et al., 2004)	596	++	++	++	--	++
Lethal factor (<i>lef</i>) gene on pXO1 plasmid	F: GGTGCGGATTTAGTTGATTC R: CCCTTTTGAATGAACTTGC (OKINAKA et al., 1999)	621	++	++	++	--	++
Edema factor (<i>cya</i>) gene on pXO1 plasmid	F: CATTAGAAAAGCAAAAAGGTG R: TCGTCTTTGTCGCCACTAIC (OKINAKA et al., 1999)	496	++	++	++	--	++
Capsule (<i>cap</i>) gene on pXO2 plasmid	F: TCCCCTTACGTAATCTGAG R: CTGAGCCATTAATCGATATG (INOUE et al., 2004)	852	--	--	--	--	++

isolate. However, the DNA from the *B. anthracis* positive control yielded the expected amplicons (Table. 1).

Sequencing and phylogenetic analysis of the *pag* gene PCR products revealed that the *pag* gene sequences of the *B. cereus* clinical isolates were 100 % similar (homologous) to the corresponding *pag* gene sequences of *B. anthracis* isolates (Fig. 1).

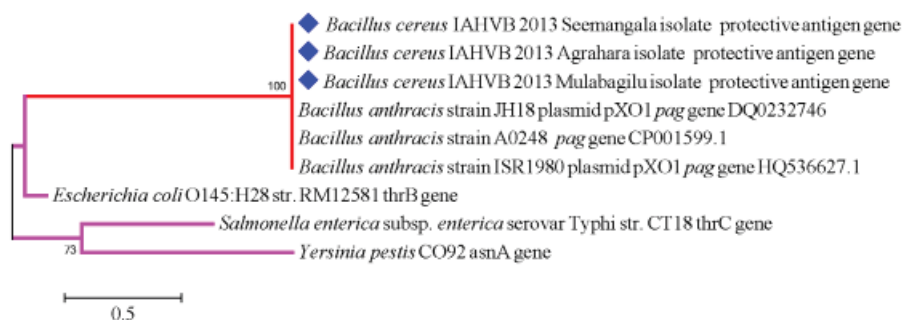


Fig. 1. Phylogenetic tree of the *pag* gene on pXO1 plasmids of *B. cereus* isolates

Mice pathogenicity test. In mice pathogenicity tests, the first passage *B. cereus* clinical isolates induced mortality of mice within 6 hours of inoculation. The heart blood and spleen samples collected upon necropsy of these mice yielded growth of pure cultures of *B. cereus*, which were positive for the pXO1 plasmid and *pag*, *lef* and *cya* toxin genes when tested by PCR, as described earlier for the original clinical isolates.

Discussion

B. cereus isolates have never been reported to cause fatal diseases in cattle. However, there are reports of *B. cereus* causing severe and often fatal pneumonia in humans (HOFFMASTER et al., 2006), African great apes (KLEE et al., 2011) and fatal septicemia in captive psittacine birds (GODOY et al., 2012). The genome sequences of *B. cereus* isolates that caused fatal disease in humans share significant homology with *B. anthracis* genome sequences and harbor almost the entire pXO1 anthrax virulence plasmid (WILSON et al., 2011). Here we report the isolation and characterization of three *B. cereus* isolates associated with a fatal anthrax-like disease in cattle, which occurred within a week in an anthrax endemic district of Karnataka State, India.

The isolates were recovered from necropsy samples of cattle in three villages viz., Seemangala, Agrahara and Mulabagilu, distanced about 10 to 15 kilometers from each other. The other common identified epidemiological link between the isolates was that these three isolates were recovered from heart blood and spleen samples of cattle that

had died with extensive vesicular lesions from FMD, rendering them recumbent, starved, dehydrated and immunosuppressed, which culminated in death.

16S rRNA gene based PCR and subsequent sequence analysis showed that the clinical isolates shared more than 99.99 % sequence identity, supporting their epidemiological assessment. Further, their phylogenetic clustering revealed a high genetic relatedness with *B. anthracis* and several other *B. cereus* and *B. thuringiensis* isolates, which augments the close genetic relationship shared by these members of the genus *Bacillus* thus resulting in their re-classification as a *B. cereus* species complex (IVANOVA et al., 2003; TOURASSE and KOLSTO, 2008).

Primers were used that were described by HOFFMASTER et al. (2006) to characterize virulent plasmids of *B. cereus* isolates that caused fatal pneumonia in welders in the USA. These primers have been shown to amplify conserved sequences of pXO1 and pXO2 plasmids in pathogenic *B. cereus*. The results of this PCR established that the three clinical isolates harbored the pathogenic pXO1 plasmid. Further, the PCR results demonstrated the presence of *pag*, *cya* and *lef* genes on the pXO1 plasmid. The sequencing and phylogenetic analysis found that the *pag* gene sequences on the *B. cereus* isolates were identical to the corresponding *pag* gene sequences on the pXO1 of *B. anthracis*. These findings justify the characteristic 'anthrax like' symptoms produced by the *B. cereus* isolates in cattle and the mortality pattern in mice.

The PCR results showed that the isolates lacked the capsule coding pXO2 plasmid, which was concurrent with the phenotypic absence of capsule expression. The findings were in accordance with the descriptions by WILSON et al. (2011) who described *B. cereus* isolates pathogenic to humans, possessing the pXO1 plasmid but lacking the pXO2 plasmid. Alternatively, it is also possible that another type of capsule might have been expressed *in-vivo* in the animal and maybe it was not possible to induce expression of this capsule type under the *in vitro* conditions that were applied during this study, in addition to the fact that it was not detected by the set of primers used in PCR. Whatever the case, a capsule negative strain could eventually cause disease in immuno-compromised cattle.

The virulence of *B. anthracis* is attributed to the production of an antiphagocytic capsule and a tripartite exotoxin. These virulence factors are plasmid encoded (HU et al., 2009; KLEE et al., 2010; WILSON et al., 2011). The plasmid pXO2 (95 kb) harbors three genes necessary for synthesis of the D-glutamyl polypeptide capsule. The structural genes for the three toxin proteins, *pag*, *cya* and *lef* are located on pXO1 (KOEHLER et al., 1993; KLEE et al., 2010). These toxins act in tandem to produce anthrax symptoms. Microbial virulence determinants are often under the control of intricate global regulatory networks. In many cases, a number of genes required for virulence are activated by one or more common environmental signals. The animals from which we were able to isolate *B. cereus* were recumbent for three to four days before death and they were under severe respiratory

distress. Similar findings were described by HOFFMASTER et al. (2006), who isolated *B. cereus* from human patients with higher frequencies of pulmonary infections. It is also intriguing that the isolates were recovered from a relatively small geographic area, where anthrax occurs naturally in herbivores. If these isolates acquired these plasmids or plasmid sequences via horizontal transfer from *B. anthracis* in the environment, it is tempting to speculate that such isolates may be restricted to or at least be more common in areas where anthrax is endemic. *B. cereus* is ubiquitous in the environment, which makes it difficult to link clinical cases to their environmental sources. Regular vaccinations against anthrax in endemic regions should now be considered seriously, taking into account not only the classical anthrax caused by *B. anthracis*, but also the 'anthrax like' disease caused by *B. cereus*.

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SAŽETAK

Bacillus cereus je posvudašnja bakterija. Većina izolata te bakterije je neškodljiva, a neki mogu uzrokovati bolesti koje se prenose hranom, infekcije rana, endokarditis, osteomijelitis, endoftalmitis i infekcije mokraćnog sustava u ljudi. Nedavno je identificirano nekoliko izolata koji uzrokuju tešku upalu pluća u čovjeka sličnu onoj kod bedrenice. Ti izolati većinom nose virulentni plazmid pXO1 vrste *B. anthracis*. U ovom radu određena su obilježja triju kliničkih izolata vrste *B. cereus* izdvojenih iz krvi sadržane u srcu i uzoraka slezene goveda uginulih pod znakovima sličnima bedrenici. Osim određivanja kulturalnih obilježja, pripremljene su i početnice za gen 16S rRNA vrste *B. cereus* koje su bile rabljene za identifikaciju izolata. Ustanovljeno je da izolati nose plazmid pXO1, a nedostaje im plazmid pXO2. Daljnja karakterizacija plazmida pXO1 pokazala je da izolati sadrže gene *pag*, *lef* i *cya* koji kodiraju za zaštitni antigen, letalni čimbenik i edemski čimbenik, toksine koji su odgovorni za pojavu bolesti u goveda slične bedrenici. Određivanje slijeda i filogenetska analiza dijela sekvencija gena *pag* izolata *B. cereus* pokazala je da su oni istovjetni sekvencijama gena *pag* na pXO1 bakterije *B. anthracis*. U testu patogenosti na miševima, izolati *B. cereus* prouzročili su njihovo uginuće šest sati nakon intraperitonejske inokulacije.

Ključne riječi: *Bacillus cereus*, plazmid pXO1, *Bacillus anthracis*, gen *pag*
