

Original Research Article

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Isolation and Identification of *Bacillus cereus* from Fish and their Handlers from Jammu, India

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ABSTRACT

The aim of the study is to know the occurrence of *B. cereus* and level of contamination with *B. cereus* in fish from Jammu region. In this study 140 samples were collected from various parts of Jammu. Out of 140 samples 44 (31.42%) samples showed characteristic peacock blue colored colonies on selective PEMBA. On Gram staining all these isolates were Gram positive rods and sometimes spores were also seen. Biochemical characterization was done to identify *B. cereus* isolates was done. Forty four (44) isolates were subjected to various biochemical tests but only 39 (27.85%) isolates showed characteristic biochemical features as that of *B. cereus*. All the 39 isolates were positive for catalase test, citrate utilization test and motility. Thirty seven (94.87%) *B. cereus* isolates hydrolyzed starch and produces beta-hemolysis on sheep blood agar. All the 39 *Bacillus cereus* isolates were positive for nitrate reduction test and Voges-Proskauer test but negative for oxidase test.

Keywords

Bacillus cereus,
Enterotoxigenic,
Fish, Gram
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Introduction

Bacillus cereus (*B. cereus*), a Gram-positive, rod shaped endospore-forming bacteria is an important cause of food-borne illness in humans and is frequently involved in food-borne outbreaks (Ryan, 2004). It is a facultative anaerobic organism belonging to genus *Bacillus*. The genus *Bacillus* is ubiquitous in nature because it does not have complex nutrient requirements. *Bacillus* group is composed of *B. cereus*, *B. anthracis*, *B. thuringiensis* and *B. mycoides* based on the similarity in 16S rRNA sequence (Seki T).

Bacterial counts in excess of 10^5 – 10^6 /g have been encountered in food suspected of causing illness. The resistance of its spores to adverse environmental conditions has enabled it to get distributed widely in the environment. Because of its ubiquitous nature, it easily spreads to the foods of plant origin and through cross contamination to other foods. The study of *B. cereus* in relation to food has gained significance in the light of its ability to form heat resistant endospores and capacity to grow and produce toxins in a

wide variety of foods. Transmission electron microscopy of the vegetative cells reveals a cytoplasmic membrane surrounding the cellular content. In addition, some strains contain an outermost crystalline surface protein (Kotiranta *et al.*, 1998, 2000). The aim of the present investigation is to study the incidence and level of contamination with *B. cereus* in fish from Jammu region.

Materials and Methods

A total of 140 samples of fish comprising of gills of fresh water fishes as such or swabs aseptically taken there from, were collected in sterile zippered plastic sachet at various wholesale and retail fish outlets from markets in Jammu city.

Isolation and identification of *B. cereus*

Isolation

Polymyxin - pyruvate - Egg yolk – Mannitol - Bromothymol blue Agar (PEMBA) media was used for isolation of *B. cereus*. The samples were processed as per the method described by Shinagawa (1990). The samples were inoculated into brain heart infusion broth (BHIB) containing polymyxin (100 units/ml). The BHIB tubes were then incubated at 37°C for 24-48 hours. After enrichment a loopful was streaked on PEMBA plates and incubated at 37°C for 24 h. The fimbriate peacock blue coloured colonies (3-5mm) surrounded by blue zone of egg yolk hydrolysis against green/greenish yellow back ground were presumed to be *B. cereus*. The presumed isolates were preserved on nutrient agar slants for further characterization.

Identification and confirmation

All presumptive colonies of *B. cereus* were purified and subjected to morphological and

biochemical tests for identification and confirmation as described in Bacteriological Analytical Manual of United States, Food and Drug Administration (Rhodehamel and Harmon 2001 and Bergey's Manual of Systemic Bacteriology (Clause and Berkeley 1986). In addition to this molecular identification of the presumptive isolates was carried out.

Morphology

Gram stained smears were prepared from slants and examined microscopically. *B. cereus* appeared as large Gram positive bacilli in short to long chains, whereas, spores were ellipsoidal, central to sub terminal and did not swell the sporangium.

Biochemical characterization of *B. cereus* isolates

Catalase test

The presumptive *B. cereus* isolates were tested for their catalase activity. Briefly, a drop of 3 percent hydrogen peroxide (H₂O₂) was taken on a clean glass slide and the presumed colony was mixed with it. The formation of gas bubbles was taken as positive reaction.

Oxidase test

A loopfull of bacterial growth was rubbed using a sterile platinum loop on oxidase disc (Hi Media Ltd., Mumbai) in a sterile Petri plate. Development of deep purple blue or mauve colour within 10 sec was considered as positive and no color change was taken as negative reaction.

Voges-Proskauer test

In a tube containing 2 ml of sterile glucose phosphate peptone water (Hi Media Ltd.,

Mumbai) a loopful of young broth culture (18-24 h) was inoculated and incubated at 37°C for 48 hrs. After 48 hrs, 0.2 ml 40 per cent potassium hydroxide was added followed by 0.6 ml of α -naphthol solution. The development of pink or crimson colour was recorded as positive reaction while no colour change was the negative reaction.

Nitrate reduction test

Sterile nitrate broth (Hi Media Ltd., Mumbai) (0.5 ml) was inoculated with a heavy growth of the test organism and incubated at 37°C for 24 hrs. Subsequently, one drop each of sulphanilic acid and the α -naphthylamine reagent was added to the test culture in broth.

The development of red colour within one minute was taken as positive reaction. The tube that did not show red colour within five minutes was treated with zinc powder and allowed to stand for 5 min. In such cases, the development of red colour indicated the presence of nitrate in the medium, as it was not reduced to nitrite by the test organism.

Starch hydrolysis test

A loopful of bacterial growth was streaked on starch agar plate and incubated at 37°C for 48 hrs. After 2 days of incubation, the plate was flooded with iodine solution. Iodine turns blue or black in the presence of starch. A clearing around the bacterial growth indicated that the organism has hydrolysed starch.

Citrate utilization test

A loopful of bacterial growth was streaked on Simmon's citrate agar slants and incubated at 37°C for 24 hrs. The development of blue colour due to utilization of citrate as a carbon source by the organism was taken as positive reaction while green colour was considered as negative reaction.

Motility test

The test was performed to observe the motility of *B. cereus* having peritrichous flagella. *B. cereus* motility medium was inoculated by stabbing down the centre with a 24 h culture suspension of presumptive isolate. The tubes were incubated for 18-24 h at 30°C and examined for diffuse type of growth away from stab line.

Haemolytic activity test

The test was performed on all *B. cereus* isolates for the production of haemolysis on sheep blood agar. A loopful of culture was streaked on blood agar plate and incubated for 24 h at 37° C and observed for haemolytic zones around the colonies. *B. cereus* cultures are usually strongly haemolytic and produced zones of complete β -haemolysis surrounding the growth.

Results and Discussion

In the present study, a total of 140 samples of fish from places in and around Jammu were analysed for the presence of *B. cereus*. Out of 140 samples 44 (31.42 %) samples showed characteristic peacock blue coloured colonies on selective PEMBA (Figure 1).

All these isolates were Gram positive rods and sometimes spores were also seen (Figures 2 and 3). Biochemical characterization to identify *B. cereus* isolates was done. Forty four (44) isolates were subjected to various biochemical tests but only 39 (27.85%) isolates showed characteristic biochemical features as that of *B. cereus*.

All the 39 isolates were positive for catalase test, citrate utilization test (Figure 4) and motile (Figure 5). 37 (94.87%) *B. cereus* isolates also hydrolyzed starch (Figure 6) and produces β -hemolysis on sheep blood agar

(Figure 7). All the 39 *B. cereus* isolates were positive for nitrate reduction test (Figure 8) and Voges-Proskauer test (Figure 9) and but negative for oxidase test.

Fig.1 *B. cereus* colonies on PEMBA



Fig.2 Gram staining of *B. cereus* (Gram positive rods)

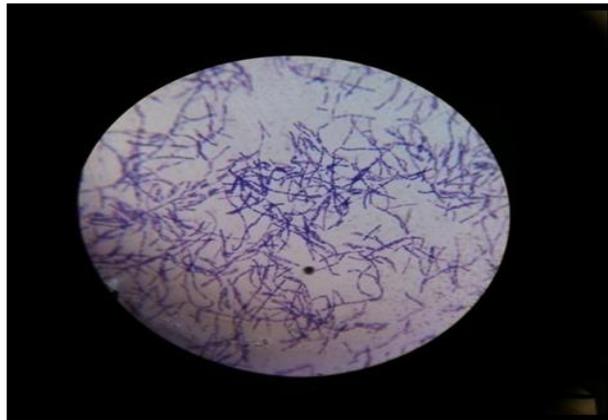


Fig.3 Spores of *B. cereus*

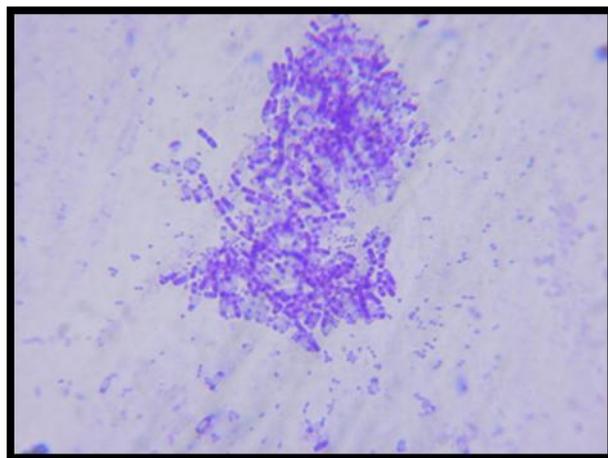


Fig.4 Color production in citrate utilization test indicates positive test)



Fig.5 Motility test (haze around stab by motile bacteria)



Fig.6 *B. cereus* showing starch hydrolysis (zone around streak)

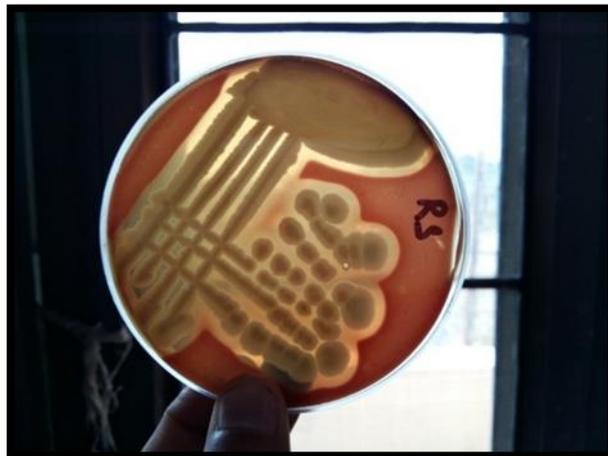


Fig.7 *B. cereus* showing β -haemolysis on sheep blood agar



Fig.8 Nitrate reduction test (brick red color indicates positive test)

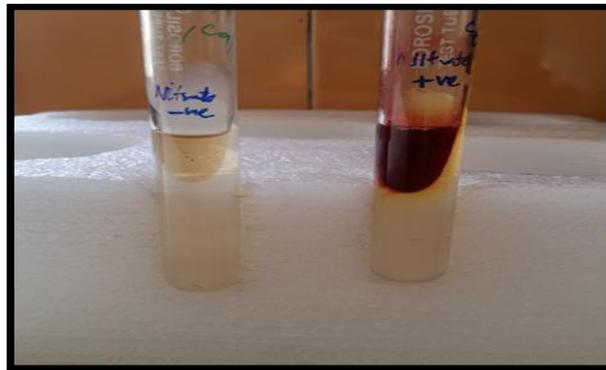
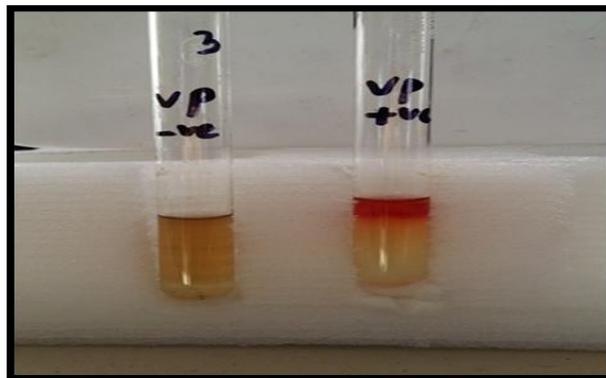


Fig.9 Voges-Proskauer test (Crimson red ring is indicative of positive test)



B. cereus is the bacterium responsible for *B. cereus* food poisoning. An estimation of 27,400 food borne cases of *B. cereus* food poisoning occurs annually in the United States. There are two forms of the intoxication: one cause's diarrhea, starting from 6 to 15 hours after consumption, and the other causes vomiting and nausea, starting from 30 minutes to 6 hours

after consumption. Symptoms in both forms last about 24 hours. Everyone is susceptible to *B. cereus* food poisoning. Some isolates of *B. cereus* can grow at refrigerated temperature (Valeroa *et al.*, 2007) and spore can survive at high temperature. This organism is known to resist even pasteurization process of milk (Novak *et al.*, 2005). In India, presence of this

organism has been reported in various food products including fish (Kamat *et al.*, 1989). Till date, three different genes viz. hbla, bceT and entFM responsible for diarrhoeal enterotoxin production in *B. cereus* have been characterized (Asano *et al.*, 1997). So far no study has been carried out to relate the presence of virulent genes and enterotoxin production in case of isolates of *B. cereus* from fish. Therefore, the present work was carried out to investigate the occurrence of enterotoxigenic *B. cereus* in fish, and also to study the relationship between diarrhoeal enterotoxin. The abilities of the spores and toxins of *B. cereus* to survive high temperatures and of certain psychotolerant strains to flourish at low temperatures make *B. cereus* a unique fish-borne pathogen. In comparison to other microorganisms, *B. cereus* is well versed with diversified characteristics like aerobic and facultative anaerobic growth, motility, psychrotrophic (4°C) and thermophilic (50°C) nature. These characteristics make *B. cereus* a unique and one of the most important food poisoning organisms. *B. cereus* makes its presence either in the form of vegetative cells or spores or toxins and is hence, anticipated from almost all foods of domestic consumption owing to its ubiquitous nature and adaptation to the environmental changes, thus posing a great public health threat. The effective use of quality control and safety measures during production/manufacture besides creating awareness among food handlers and consumers becomes necessary to ward off the menace of *B. cereus* and other food poisoning organisms.

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