ISSN 0975-6299 Vol 3/Issue 2/April – June 2012

International Journal of Pharma and Bio Sciences

RESEARCH ARTICLE MICROBIOLOGY

STUDY OF ENTEROTOXIGENICITY OF *B. CEREUS* EMETIC STRAIN BY SKIN VASOPERMEABILITY REACTION IN RABBITS AND POULTRY

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ABSTRACT

The present study pertains to the toxigenicity of *Bacillus cereus* emetic strain isolated from raw milk and milk products in Kashmir valley. The study was conducted during October 2009-June 2010. A total No. of 175 samples comprising of 50 raw milk and 125 of different milk products were tested. *Bacillus cereus* emetic strains were isolated from 8 of the raw milk and 20 of the milk product samples, (6, 5, 4, 3 and 2 samples out of 25 each of rasgulla, burfi, ice-cream, rasmalai and cheese/paneer, respectively). *B. cereus* emetic strains were isolated from 16 percent of the raw milk. Among the different milk products analysed *B. cereus* emetic strains could be isolated from 20, 24, 12, 16 and 8 percent of burfi, rasgulla, rasmalai, ice-cream and cheese/paneer, respectively. The field isolates and the standard strain of *Bacillus cereus* had similar cultural, morphological and biochemical characteristics. Raw milk, burfi and ice-cream revealed biotypes 2, 5 and 7, whereas, rasmalai and cheese/paneer revealed only biotype 5. Biotypes 2 and 5 were recovered from rasgulla. *Bacillus cereus* emetic enterotoxin (BCEET) produced by field isolates and the standard strain produced comparable vascular permeability reaction on intradermal inoculation. The development of moderate to severe VPR was observed in rabbit skin, where as there was mild VPR in poultry skin.

KEY WORDS

Bacillus cereus emetic strains, raw milk, milk products, *Bacillus cereus* emetic enterotoxin (BCEET), vascular permeability reaction (VPR).

INTRODUCTION

Bacillus cereus, a gram positive, spore forming bacterium is wide spread in environment (soil, water and dust), and easily contaminates the foods of both plant and animal origin such as cereals, vegetables, milk and milk products, meat and meat products etc, there by causing food borne illnesses in humans [1].

Outbreaks of food poisoning due to *Bacillus cereus* have been described since the beginning of the last century with the first confirmed report in Norway in 1948 [2]. Since then many food-borne outbreaks were reported [3, 4]. Milk, being ideal medium for growth of microorganisms, makes it suitable for multiplication of *B. cereus* as well as elucidation of its toxin in it. With the advent of increased number of psychrotolerant *B. cereus* strains, the dairy industry has witnessed increased reports of food poisoning outbreaks due to this organism [5].

Two distinct types of gastrointestinal disorders caused by *B. cereus* in humans viz; an early "emetic syndrome" and a late onset "diarrheal syndrome" involving two different types of enterotoxins, have been recognized [6]. The emetic syndrome, a food borne intoxication, caused by preformed *B. cereus* emetic enterotoxin (BCEET) in food has a rapid onset (1-5 hours) characterized predominantly by nausea and vomition, resembling closely to staphylococcal food poisoning [7]. In contrast the diarrhoeal syndrome is caused due to production of *B. cereus* diarrhoeal enterotoxins (BCDET) during the vegetative growth of bacteria in the foods or in the intestines following ingestion, has a longer incubation period (12-24 hours) and is characterized by symptoms
like diarrhoea, abdominal pain and rectal tenesmus, resembling closely *Clostridium perfringens* type A food poisoning [6, 7].

Apart from gasteroenteritis *B. cereus* is also involved in a variety of non-GIT infections like meningitis, endopthalmitis, endocarditis, periodonitis, osteomyelitis, wound infection and septicaemia in humans [8]. It is also emerging as potential pathogen of serious concern in animals owing to increased reports of its role in diseases like, osteomyelitis, middle ear infections, abortions and mastitis [9]. These reports unfold its explosive pathogenic role in various infections of animals.

The biological effects of *B. cereus* toxins have been studied extensively. The diarrhoeal enterotoxin produced during the exponential growth phase of the organism is destroyed at 56^{o}C in 20 minutes, a temperature which is far less than the temperature attained in conventional cooking. However, emetic enterotoxin, commonly called as cerulide, produced in the stationary growth phase, is highly heat stable (126^{o}C for 90 minutes), withstanding extremes of pH (2-11) and is unaffected by the temperature attained during conventional cooking process. As a result the episodes of food poisoning out breaks due to *B. cereus* emetic toxin outnumber the diarrhoeal ones.

The production of enterotoxins by *B. cereus* is dictated by the type of food rather than the strain involved. Studies on food ingredients have indicated rice and rice flour containing a high percentage of *B. cereus* emetic strains whereas diarrhoeal strains are found in almost all foods of animal origin. During growth, harvesting, milling, and other agricultural
operations rice can variably become contaminated with *B. cereus* spores from a wide variety of environmental sources including soil,

dust, sediment, and water [10]. The spores survive normal cooking temperatures [11] and proliferate when the cooked rice is stored at room temperatures for long time leading to the episodes of intoxication (emetic type) or toxi-infection (diarrhoeal type) due to consumption of such temperature-abused rice. The emetic enterotoxin is selectively produced in rice and vegetable sprouts, milk and milk products, the later mostly being adulterated by the addition of rice powder/flour. The long transportation associated with continuous shaking of milk from rural areas to urban consumption sites provides an excellent shake culture media for the production of *B. cereus* emetic enterotoxin. Keeping in view the importance of *B. cereus* in foodborne infections and intoxications, the present study was undertaken.

MATERIALS AND METHODS

(i) Sampling

A total of fifty samples of market milk (100 ml each) were collected in sterile urocols from local milk vendors of five arbitrary zones of Srinagar city viz. north (Hazratbal and surrounding areas), south (Sonawar and Dalgate), east (Harwan, Shalimar and Nishat), west (Qamarwari and adjacent areas) and central (Habbakadal and adjacent areas). The samples (**Table 1**) were brought to the laboratory on ice and processed within two hours of collection. A total of 125 samples of milk products, comprising of Burfi, Rasgola (white), Rasmalai, Ice cream, and Cheese (25 each) were collected aseptically from various retail outlets of the afore said zones of the Srinagar city (**Table 1)** The samples were brought to the laboratory on ice and processed within two hours of collection.

Table 1

Source and detail of milk and milk products collected from different zones of Srinagar city.

Sample	Central	Eastern	Western	Northern	Southern	Total
Raw milk	10	10	10	10	10	50
Burfi	5	5	5	5	5	25
Rasgola (white)	5	5	5	5	5	25
Rasmalai	5	5	5	5	5	25
Cheese/paneer	5	5	5	5	5	25
Ice cream	5	5	5	5	5	25
Total	35	35	35	35	35	175

(ii) Standard Bacillus cereus strain

The standard *Bacillus cereus* emetic strain (NCTC 11143) originally recovered from a natural emetic type food poisoning outbreak by Public Health Laboratory Service Coollindale, London was obtained from Department of Veterinary Public Health, Ranchi Veterinary College, Ranchi, Bihar.

(iii) Production of Bacillus cereus Emetic enterotoxin (BCEET)

Bacillus cereus emetic enterotoxin was produced as per the protocol of [12]. The standard strain (NCTC 11143) and field isolates of *B. cereus* were plated on MYPA plates and incubated at 37^{o}C for 12 hours. A colony was inoculated into 15 ml BHIB and incubated at 37^{o}C for 8 hours. One milliliter of this preculture was inoculated into 100 ml of the rice culture broth (RCB) and cultured at 30^{o}C for 15 hours on a shaker. Alpha amylase (25.0 mg/100ml) was added to RCB followed by further incubation for 30 minutes at 25^{o}C on a shaker to facilitate hydrolysis. The culture was then centrifuged at 4500 rpm for 40 minutes. The clear supernatant was collected and filtered through seitz filter and then boiled at 100 ^{o}C.

This was designated as *B. cereus* emetic enterotoxin (BCEET). The BCEET was concentrated to 1/10th of its volume by boiling on a water bath at 100 ^{o}C for about 10 minutes. The protein concentration of the emetic entertoxin recovered was estimated by the method of [13]. The toxin was stored under refrigeration till further use.

(iv) Vascular permeability reaction

The toxigenicity of BCEET was observed by demonstration of Vascular permeability reaction (VPR) in Rabbit and poultry skin as per the method described by [14]. About 0.05 ml of concentrated BCEET from standard *Bacillus cereus* emetic strain NCTC 11143 was injected intradermally into clean shaved abdominal area of two adult rabbits and de-feathered area of breast of two poultry birds. Adjacent to these sites, an equal amount of concentrated BCEET from the *B.cereus* biotypes understudy was also injected. Filtrate of uninoculated RCB was also injected adjacent to these sites as control. After three hours of inoculation, 2.5 ml of 0.25 percent trypan blue dye in Normal Saline Solution was injected I/V in to the ear vein of each of the rabbits and wing veins of poultry. An hour after the adminstration of the dye, vascular permeability reaction was observed as zones of light and dark blue areas surrounding the point of inoculation. Gross observations like intensity of colour change, presence of oedema area affected etc were recorded at varying time intervals of 3, 6 and 12 hours post inoculation (PI).

RESULTS

Production and concentration of Bacillus cereus Emetic enterotoxin (BCEET)

The most common biotypes recovered from milk and milk products (Barfi, Rasgulla, Rasmalai, Ice-cream and Cheese), viz. biotype 2, 5, 7 and also the standard *Bacillus cereus* emetic strain NCTC 11143 were used for the production of emetic enterotoxin. The protein concentration of the emetic enterotroxins recovered the isolates under study was estimated by the method of [13], results of which are presented in the **Table 2**. The enterotoxins were concentrated by boiling on a water bath at 100 ^{o}C till the reduction of its volume to 1/10 th, before being used for Vascular permeability reaction in Rabbit and poultry skins.

Table 2
***Protein concentration of B. cereus emetic enterotoxin produced different field isolates and standard NCTC 11143 strain*.**

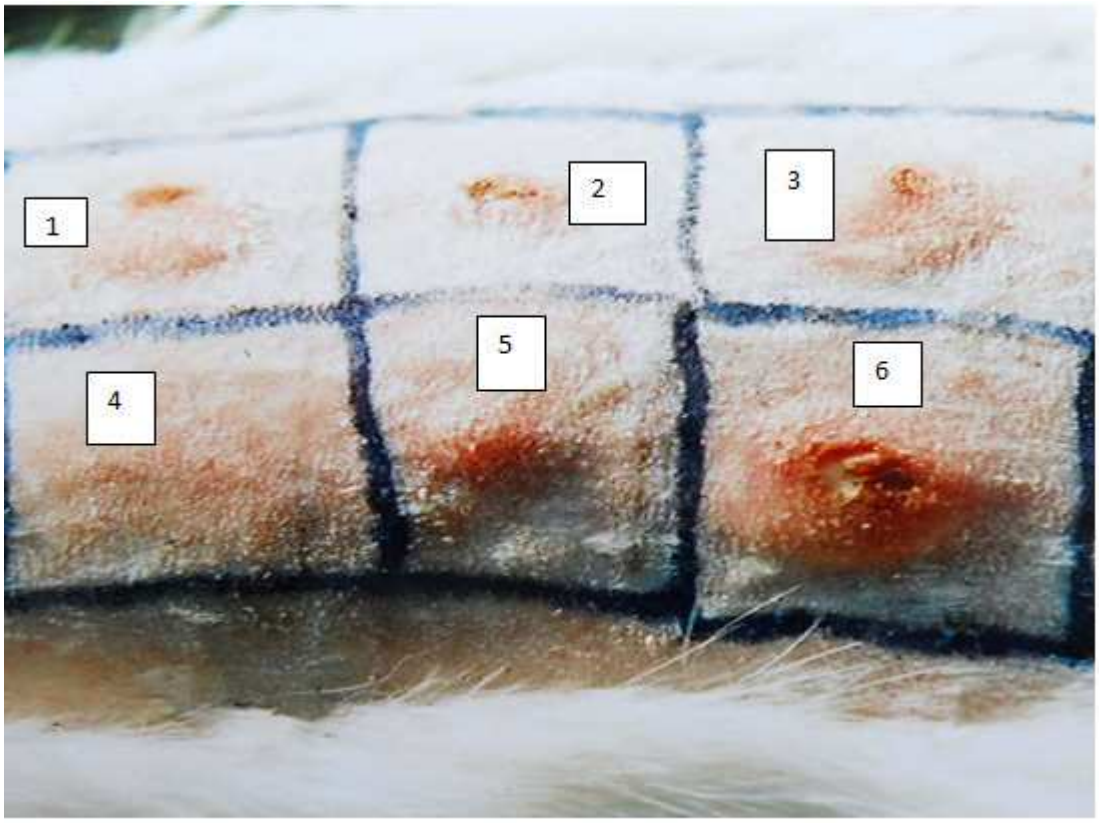
S. No.	Isolate no.	Biotype	Protein conc. of BCEET (ug/ml).
1.	NCTC 11143	1	800
2.	Rg 9	2	500
3.	Rm 7	5	600
4.	Br 23	7	500
5.	Concentrated RCB	__	00.00

Vascular permeability reaction

The field isolates were compared with the standard NCTC 11143 strain, with respect to production of emetic enterotoxin and their biological effects on rabbit and poultry skins.

The VPR was more pronounced in rabbit skin where the inflammatory changes could easily be measured against poultry skin which presented a mild reaction with similar doses. VPR zones were pronounced following intradermal inoculation of BCEET by 12 hours and ranged between 8 and 11.5 mm in diameter. The higher

VPR zone was presented by BCEET isolate no.Mj 8 recovered from milk and the lowest zone by BCEET from isolate no. Rm 11 recovered from rasmalai. The BCEET from the standard NCTC 11143 strain produced a severe vascular permeability reaction in 12 hours following I/D inoculation. The RCB control points, on the other hand showed no inflammatory reaction after 48 hours of I/D inoculation. The results are presented in**Figure 1**



1.BCEET(MJ22) 2. BCEET(Br 7) 3. BCEET(Rm 10) 4.RCB(control) 5. BCEET(Rg 14) 6. BCEET(NCTC -11143)

Figure 1
Shows Vascular Permeability Reaction in rabbit skin.

DISCUSSION

Milk and milk products are naturally contaminated with diarrhoeal strains of *Bacillus cereus*, which on conventional heating are easily killed as is true with other such food poisoning causing organisms. The presence of emetic strains of *B. cereus* in milk and of emetic enterotoxins is anticipated in situations where milk is adulterated with starchy foods like rice powder etc.

BCEET recovered from the field isolates and from the standard emetic strain produced comparable biological effects in the rabbit and poultry skins. 0.05 ml of the concentrated BCEET in normal saline solution produced moderate Vascular Permeability Reaction which has been attributed to the release of 5 HT by the previous workers [14], as assessed by the gross examination of the intensity and size of the blue patch produced as a result of the exuded dye-albumin complex at the site of the intradermal injection of the BCEET. Method to

study the effects of BCEET in cell cultures have been reported with vacuolation and rounding. However a more convenient and easy method to demonstrate the vasculo permeability reaction following I/D inoculation was demonstrated in the present study. Many workers have also reported extensive inflammatory reaction with severe necrosis in rabbit skin following I/D inoculation of BCEET [14, 15]. In the present study however, more severe reactions were observed in rabbit skin than in the poultry which were more defined and reproducible. Therefore, use of rabbit skin for demonstration of VPR by BCEET seems to be a better option.

Six isolates from the most common biotypes from milk and milk products along with standard *Bacillus cereus* strain (NCTC 11143) were cultured to produce emetic enterotoxin by shake culture method using rice culture broth as a basal medium. The nature and type of food was related to the amount of enterotoxin production by many workers [14, 16]. This is also substantiated by the fact that food poisoning outbreaks due to *Bacillus cereus* are restricted to the consumption of particular food items more so in case of cooked rice where emetic strains are predominantly found and frequently produce emetic enterotoxins.

CONCLUSION

In the present experiment, we conclude that rabbit skin is more defined and reproducible than in the poultry. Therefore, use of rabbit skin for demonstration of VPR by BCEET seems to be a better option.

ACKNOWLEDGEMENT

The authors thanks to the Dean, FVSc and A.H, Shuhama, Alusteng, Srinagar,for providing necessary facilities at time of research.

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