Bacillus cereus in Ready-to-Eat Foods Available in Kathmandu, Nepal

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ABSTRACT

Objectives: The aim of this study was to assess the ready-to-eat food samples available in Kathmandu and detect if *Bacillus cereus* were present.

Methods: A cross sectional study comprising of field and laboratory was conducted in Kathmandu, Nepal. A total of 240 ready-to-eat food samples were collected from February 2021 to January 2022 covering different food outlets of Kathmandu. The food samples were grouped into three main categories- rice, bakery, and dairy. The homogenate mixture of sample was prepared. The samples were processed following the standard microbiological procedures and *Bacillus cereus* was identified by different biochemical tests.

Results: Out of 240 samples analyzed, 40% samples showed the presence of *Bacillus cereus* which was most prevalent in bakery items followed by rice and dairy items (50%, 42.5%, and 28% respectively). One of the *Bacillus cereus* isolates was emetic toxin-producing type as detected from the phenotypic analysis. The contamination of *Bacillus cereus* ranged from 1.64 to 6.83 log cfu/g and 20.8% of the samples were potentially hazardous (>5 log cfu/g).

Conclusion: This study reports relatively high prevalence of *Bacillus cereus* in ready-to-eat food samples indicating a serious public health concern. There may be a likely connection between the standards maintained during preparation, packaging, storage, and transport; and regular monitoring of ready-to-eat food items is imperative to prevent any food poisoning incidences.

Keywords: *Bacillus cereus*, toxin, food poisoning, contamination, ready-to-eat

INTRODUCTION

In developing and under-developed countries, foodborne pathogenic microorganisms are found to be the main cause of illness and even death leading to the expenditure of billions of dollars in medical treatment and social care. The contributing factors are the changes in eating habits of people, mass catering complex, lengthy food supply Chains with more international movement and inadequate hygienic practices. The production, sale and consumption of ready-to-eat (RTE) food items is in an increasing trend because of their easy availability and convenience despite the fact that it may be a source of food borne diseases if it is not processed and handled properly (Mensah et al, 2012).

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Bacillus cereus is a group of ubiquitous aerobic or facultative anaerobic, spore-forming, gram positive, motile, rod-shaped, mesophilic bacteria, growing optimally between 20-40^{0}C, normally present in soil as saprophyte (Vilian 2006). The spores are frequently found to contaminate varieties of food including meat, eggs, dairy products, rice, cereals, dried foods, spices and vegetables (Tallent 2012). *Bacillus cereus* causes two different types of food poisoning- diarrhoeal and emetic type and is one of the most common cause of "fried rice syndrome". The diarrhoeal type of food poisoning is caused by enterotoxin produced in the small intestine during the vegetative growth of *Bacillus cereus* (Ross 2019). Emetic type of food poisoning is due to the intoxication caused by heat stable, acid and alkali resistant, proteolytic toxin (Fagerlund 2010). *Bacillus cereus* food poisoning has been reported in different countries from cereals, spices, cooked food, fresh-cut vegetables, raw and cooked poultry meats, sea foods, canned foods, pastry and dairy products (Rosenquist 2005; Singh 2015; Abraha 2017).

The presence of environmental contaminants, pathogenic bacteria and disregard of good hygienic practices (GHPs) and good manufacturing practices (GMPs) promotes the rapid proliferation of pathogenic bacteria in foods. Most of the vendors, especially the cooks and serving personnel, are uneducated and untrained in food hygiene and sanitary practices, the working conditions are poor and they have almost no knowledge about the causes of foodborne diseases and the fact that they can be the carriers of foodborne pathogens (Barro, et al., 2007).

The water activity of fried rice has been reported to range from 0.912 to 0.961 which supports the growth of *B. cereus* (minimum working limit- 0.912) (Ankolekar, 2009). The number of *B. cereus* in boiled rice held at 30^{0}C can double in 25-60 minutes. About 14-43% of people have been reported to be asymptomatic carriers of *B. cereus*. During the acute phase of foodborne and related illnesses, up to 10^9cfu/g of *B. cereus* can be present in feces and the bacteria can also be isolated from vomitus. Hence the human contacts who are in constant exposure to food during preparation, handling, and serving can be an important source of *B. cereus* in foods (Jenson & Moir, 2003). However, the prominent belief is that the point of entry of *B. cereus* in food chain is contaminated soil or water.

Very few studies of food poisoning have been conducted in our country and most studies focus on bacterial agents other than *Bacillus cereus*; therefore we considered to study the occurrence *Bacillus cereus* in foods.

MATERIALS AND METHODS

Study Design, Study Site and Sample Size

The study was quantitative, and primary data were collected from February 2021 to January 2022. The variables of this study were the occurrence of *B. cereus*, their ability to produce enterotoxin and emetic toxin. The study was cross-sectional comprising of field and laboratory based procedures. Ready-to-eat food samples (n=240) were collected from different departmental stores, restaurants, hotels, grocery shops and street food stalls mainly from the central areas of Kathmandu district like Naradevi, Ason, Ratnapark, Bagbazar, Putalisadak, Dillibazar and Mahaboudha.

Sample collection and transportation

Packaged foods were transported to the laboratory of Tri-Chandra Multiple Campus immediately. Cooked food like rice items were requested as takeaways. The samples were transported in ice box and processed immediately, in case of delay, they were kept in refrigerator at 4^{0}C.

Isolation and enumeration of *B. cereus*

The homogenate mixture of sample was prepared by adding 50g of sample into 450ml of phosphate buffer saline and blended for 2 minutes at 10,000 to 12,000 rpm. Enumeration of bacterial load in the homogenate sample was done up to 10^{-6} (1:10 dilution) in dilution blank. The sample and the diluent were homogenously mixed by vortexing. For plate count in Mannitol Egg Yolk Polymyxin (MEYP) Agar, samples were inoculated in duplicate by spread plate method and incubation was done at 30^{0}C for 18-24 hours. After incubation, media turned pink due to mannitol non-fermenting colonies. The pink colonies surrounded by precipitate zone gave an indication of the presence of *Bacillus cereus*. Such typical white opalescent colonies were counted to determine the bacterial load.

Further confirmatory tests were done after sub-culturing in Nutrient Broth (NB)/ Nutrient Agar (NA) for 4 hours at 30^{0}C. Gram staining, spore staining were performed followed by other biochemical tests- catalase, oxidase, motility, Voges Proskauer (VP), Citrate Utilization, Oxidative-Fermentative (O/F), Nitrate Reduction and also checked for the growth in trypticase soy blood agar (with beta haemolysis), phenol red glucose broth and lysozyme broth.

RESULTS

Food samples analyzed

There were three main categories of ready-to-eat food samples analyzed in this study. These food samples were rice items, dairy items and bakery items. The individual food items are listed in (Table 1).

Occurrence of *Bacillus cereus*

Out of 240 ready-to-eat food samples collected from different food outlets located in Kathmandu, *Bacillus cereus* was isolated from 96 samples (40%) (Figure 1). As shown in Table 2, *Bacillus cereus* was most prevalent in bakery items followed by rice dishes and dairy items (50%, 42.5%, & 28% respectively). Vegetable fried rice, bakery items with meat products (like chicken pizza and chicken patties) and butter were found to be highly contaminated with *B. cereus* than other rice, bakery and dairy items respectively (Table 3). One isolate of *Bacillus cereus* from cheese sample was emetic toxin-producing type, and 95 were enterotoxin-producing (98.96%).

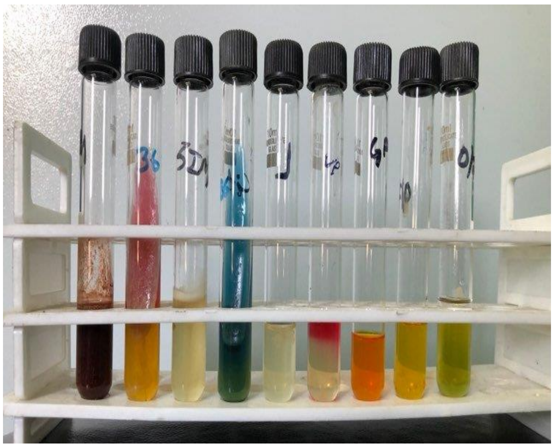
Enumeration of *B. cereus* in different varieties of ready-to-eat food samples

The contamination of *Bacillus cereus* ranged from 1.64 to 6.83 log cfu/g and 20.8% (n=50) of ready-to-eat food samples were found to be potentially hazardous (>5 log cfu/g). Among 80 samples of each of the 3 food categories, 19 (23.75%) rice items, 29 (36.25%) bakery items and 2 (2.5%) dairy items were categorized as potentially hazardous. Others were categorized as unsatisfactory, borderline or satisfactory on the basis of the load of *Bacillus cereus*. The categorization was done on the basis of the count of *B. cereus* (FSAI 2001).

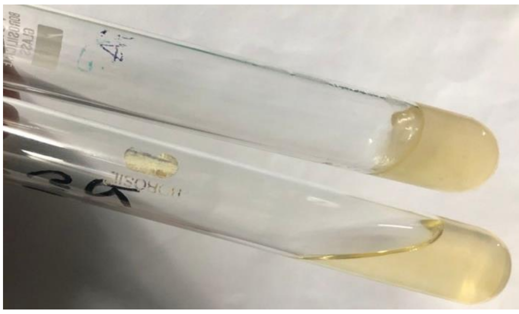
Overall categorization of food samples

Among 240 ready-to-eat food samples analyzed for *B. cereus* contamination, 20.83% (50/240) samples were categorized as potentially dangerous, 6.67% (16/240) categorized as unsatisfactory, 8.75% (21/240) as borderline and the remaining 63.75% (153/240) as satisfactory (Figure 3).

Photograph 1: *Bacillus cereus* in MEYP agar

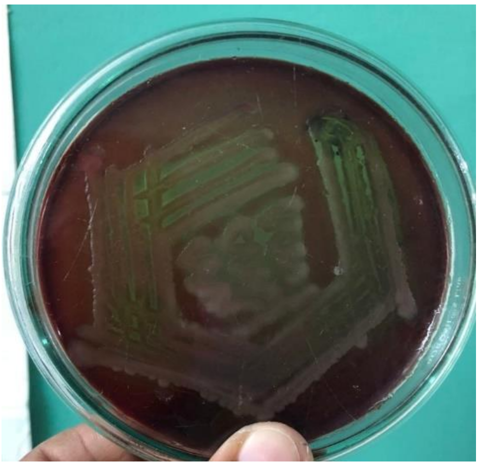


Photograph 2: Biochemical tests (from Left to Right): Nitrate positive, TSI Alk/A, G- ; SIM H_2S Negative, Indole Negative, Motile; Citrate Positive; Lysozyme Positive; VP Positive; Phenol Red Glucose Broth Positive, Fermentative.



Photograph 3: Gelatin Liquefaction Test. Emetic or enterotoxin strains were detected on the basis of biochemical tests by their ability to degrade starch and hemolysis on blood agar.

Photograph 4: Starch Hydrolysis Positive



Photograph 5: Haemolysis on Blood Agar

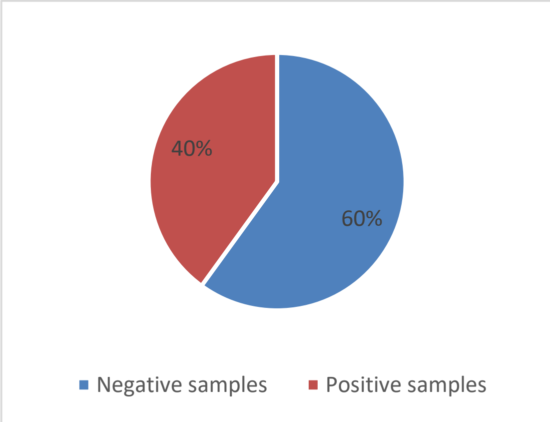


Figure 1: Prevalence of *B. cereus* in ready-to-eat food samples

Table 1: Varieties of ready-to-eat food samples

Categories	Individual food items
Rice items	Buff Biryani, Buff Fried rice, Chicken Fried rice, Veg Fried rice, Plain rice
Bakery items	Bread-like items- Bread, Bun, Chocolate Danish, Plain Doughnut, Fruit Cake, Wheat rusk Bakery items with cream/cheese- Cream Roll, Croissant, Cream Doughnut, Swiss Roll, Cheese patties, Cheese pizza Bakery items with meat- Chicken burger/patties/pizza Bakery items with vegetables- Veg burger/patties/pizza Roti-like items- Lachha paratha, Pizza roti, Plain roti
Dairy items	Butter, Cheese (Regular, Mozarella, Paneer), Cream, Ghee, Yoghurt

Table 2: Distribution of *B. cereus* among different categories of ready-to- eat food samples

Category of Ready-to-eat food items	**Total no. of samples**	**Total no. of samples showing growth of *B. cereus* (%)**
Rice dishes	80	34 (35.42%)
Bakery items	80	40 (41.67%)
Dairy items	80	22 (22.92%)
Grand Total	240	96 (100%)

Table 3: Distribution of *B. cereus* among five different categories of three food items

Ready-to-eat food items	**No. of samples**	**No. of positive samples**	**Percentage**
Rice			
Buff Biryani	4	1	25%
Buff Fried rice	4	1	25%
Chicken Fried rice	34	10	29%
Veg Fried rice	34	20	59%
Plain rice	4	2	50%
Total rice items	80	34	43%
Bakery items			
Bread-like items	18	10	56%
Bakery with cream/cheese	16	6	38%
Bakery with meat products	20	12	60%
Bakery with veg products	14	6	43%
Roti-like items	12	6	50%
Total bakery items	80	40	50%
Dairy items			
Butter	6	3	50%
Cheese	52	19	37%
Cream	6	0	0%
Ghee	6	0	0%
Yoghurt	10	0	0%
Total dairy items	80	22	28%

Table 4: Microbiological quality of different ready-to-eat food items

Category of ready-to-eat food items	Satisfactory $<10^3$ cfu/g n (%)	Borderline 10^3 - $<10^4$ cfu/g n (%)	Unsatisfactory 10^4 - $<10^5$ cfu/g n (%)	Potentially Hazardous $\geq 10^5$ cfu/g n (%)
Rice dishes (N=80)	46 (57.5)	9 (11.25)	6 (7.5)	19 (23.75)
Bakery items (N=80)	45 (56.25)	-	6 (7.5)	29 (36.25)
Dairy items (N=80)	62 (77.5)	12 (15)	4 (5)	2 (2.5)

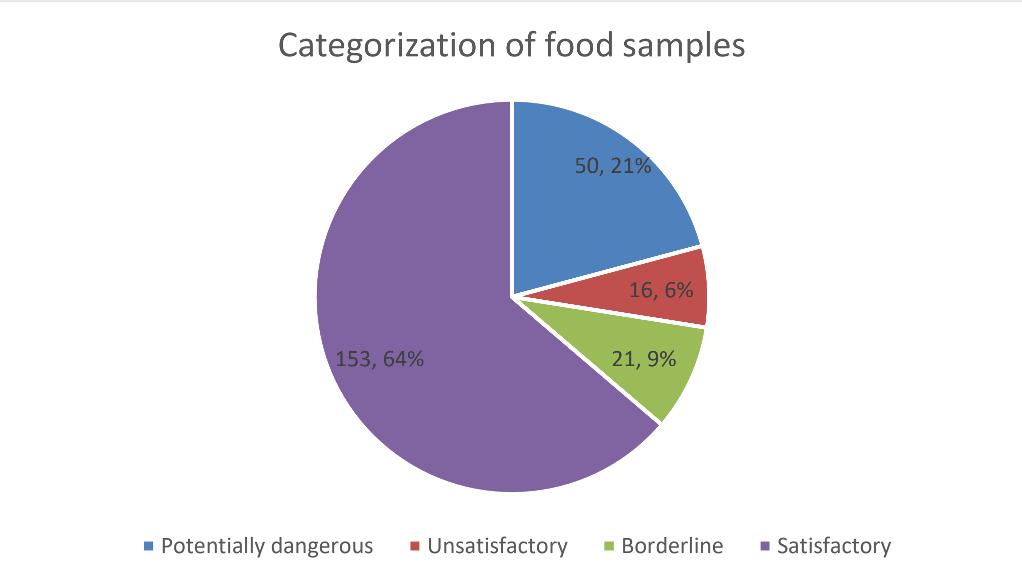


Figure 3: Overall categorization of microbiological quality of food samples tested

DISCUSSION

Among 240 samples analyzed, 60% of the samples showed no growth of *B. cereus* which might be due to the relative absence of *B. cereus* in the sample. This indicates that these food items were sampled when fresh and had not been handled excessively or exposed for long hours (Yebodah-Manu et al, 2010). As *B. cereus* is commonly found in soil and growing plants, it is mostly isolated from foods of Plant origin. Vegetables and other crops are irrigated using polluted water, a high level of contamination is expected if the vegetables are not washed thoroughly (Mensah et al, 2007). During the preparation of rice dishes, the vendors left the raw materials open, which can lead to exposure to dust particles that might contain bacterial cells and spores. The vegetables or meat were chopped and usually used without proper washing.

In a study of un-stored vegetable purees pasteurized in their final packages, *B. cereus* was detected in 20% of the samples in the range of <10 cfu/gm (Choma, et al., 2000). We also detected the presence of *B. cereus* in vegetarian foods like fried rice, patties and pizza.

Other sources of contamination of *B. cereus* are dirty hands or cooking/cutting utensils like knives, the practice of handling foods without using gloves and contamination by flies and other insects (Singleton, 2004). Prolonged holding at ambient temperatures and defective storage temperatures affect the microbiological quality of food (Mepba et al, 2007). Very few vendors used refrigerators for storing the boiled rice before preparing fried rice. Common practice of refrigerating poultry and meat before cooking was observed but not stored vegetables and boiled rice.

It was observed that the vendors did not use gloves during preparation and serving of fried rice or while serving or selling the unpacked bakery items or dairy items. Poor hygiene and poor sanitary practices was observed in most of the vendors and restaurants serving fried rice. A study conducted in Kathmandu district in 2015 which analyzed about 52 fried rice samples reported similar unhygienic practices among the vendors (Yadav, 2016).

A significant safety risk is associated with the consumption of rice products due to the frequent occurrence of *B. cereus* as shown by different studies. 61 samples of raw rice tested in Spain showed that unhusked rice had higher *B. cereus* count ranging from 1.2-3.5x 10^3 cfu/g than in husked rice samples (Sarrias et al, 2002); in the range of 3.63 to 7.31 log cfu/gm in ready-to-eat rolled cooked rice (kimbap) (Bahk et al, 2007); >10^4 cfu/gm in about 20% of the raw rice samples (Fang et al, 2003). *B. cereus* was present in all the samples tested in Pakistan with relatively low counts of the bacteria ranging from 3.34 to 0.933 log cfu/ml (Tahir et al, 2012).

In Hyderabad, India, average microbial load of *B. cereus* was 3x10^5 cfu/gm in chicken fried rice (Sudershan et al, 2012); present in only 7 samples (1%) of take-away cooked rice and chicken sandwiches with count greater than 10^4 cfu/gm (Little et al, 2002). The higher counts in our study (40%) might be because the rice used to prepare fried rice or biryani was boiled in advance (a day or two earlier) and stored mostly at room temperature. This practice must have allowed the already existing spores of *Bacillus cereus* to germinate easily. Preparing boiled rice in large quantities requires longer time to cool down, worsening the situation.

The rice dishes served in early hours, is usually cooked at dawn and displayed in open containers. This can easily lead to the contamination with *B. cereus* with bacterial counts enough to cause foodborne illnesses (Granum, 2007). The presence of *B. cereus* in different types of rice dishes and other ready-to-eat food items can be attributed to its presence in the raw material itself in the form of spores which has the ability to withstand the temperature of cooking in addition to the possibility of re-introduction of the spores, and also the vegetative cells, into the food due to post-cooking contamination. The presence of *B. cereus* in the food is of great significance because of its ability to produce heat sensitive and heat stable toxins responsible for different types of food poisoning (Sudershan et al, 2012).

The growth temperature of *B. cereus* ranges from 4-50^{0}C (Guinebretiere, et al., 2008), the practice of keeping ready-to-eat foods in room temperature unfortunately supports its growth.

A survey of street foods conducted in Kathmandu showed that the frequency of detection of *Bacillus cereus* was 1.96% (Tuladhar and Singh, 2012). Yadav (2016), Ghosh et al (2007) and Wogu et al (2011) showed that *B. cereus* was one of the most prevalent bacteria in food products. Ankolekar (2009) found that in addition to *B. cereus* (46.6%), 6.1% of samples were positive for *B. thuringiensis*. We also observed characteristic fried-egg type colonies of *B. thuringiensis* but detailed analysis was not within the scope.

All the ready-to-eat food samples analyzed were categorized according to FSAI (2011). The results of *B. cereus* counts ranged from 1.64 to 6.83 log cfu/g and 20.8% (50/240) of ready-to-eat food samples were categorized as potentially hazardous (>5 log cfu/g), 6.67% as unsatisfactory, 8.75% as borderline and the remaining 63.75% as satisfactory. The food items falling in potentially dangerous or unsatisfactory categories are beyond the microbiological standard, indicating poor hygiene, improper/unhealthy practice of handling and processing, cross contamination or the possible survival of bacteria or their spores present in food prior to processing.

The emetic strains of *B. cereus* are unable to degrade starch, unable to ferment salicin, they do not possess haemolysin BL (Hbl) encoding genes and show very weak or no hemolysis whereas those strains possessing enterotoxins and are isolated from different diarrhoeal outbreaks and foods show haemolysis in blood agar, produce Hbl and degrade starch (Ehling-Schulz, et al., 2005). We identified 1 out of 96 *B. cereus* isolates as emetic type and the rest 95 were enterotoxin producing type. Altayar (2005) also isolated a single out of 148 emetic toxin producing strain of *B. cereus*.

Earlier, starchy foods like pasta or rice were found to be more responsible for food intoxications with emetic *B. cereus*, however, recent studies have shown growing evidence that emetic *B. cereus* are more volatile (Dietrich, 2021). This is in accordance to our study; *B. cereus*, more famously known to cause "fried rice syndrome" has been isolated not only from rice items but also from bakery and dairy products.

B. cereus was reported in 48% of pasteurized milk in Netherlands and contamination level ranged from 10^2 to 10^6 cfu/gm (Notermans, et al., 1997); in 14% and 42% of powdered infant formula (PIF), posing greater risk to infants (Zhuang, et al., 2019). Our study showed the presence of *Bacillus cereus* in 22 of 80 dairy products (27.5%). A serious food poisoning incidence occurred in Brussels, Belgium and a young male died after consuming spaghetti meal contaminated with *B. cereus*. The sample contained 14.8 µg/gm of emetic toxin cereulide (Naranjo, et al., 2011). The fatal consequence of the presence of *B. cereus* in food items cannot be undermined. Cautions should be exercised at all steps of food preparation and handling so that such occurrences are prevented.

Future studies should be directed towards expanding the range of samples and including wider geographic locations. Study should also focus on other toxin producing food poisoning bacteria and the detection of toxin producing genes and toxin level.

CONCLUSION

Significant presence of *B. cereus* in ready-to-eat food items (40%) was found; 20.8% (50/240) of the samples were categorized as potentially hazardous. Both Emetic and enterotoxin-producing types were detected. It is imperative to maintain appropriate standards during the preparation, packaging, storage, and transport and regular monitoring of ready-to-eat food items to prevent food poisoning incidences.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this paper.

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