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Incidence of multiple drug resistant *Bacillus cereus* in some popular snacks and sweets sold in Kolkata city, India

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Abstract

Background: Snacks and sweets from road side eateries are cheap sources of nutrition but occasionally cause numerous public health hazards most importantly gastroenteritis. Various factors including poor infrastructure to prepare foods, post-preparation contamination during storage and handling as well as unhygienic surroundings may contaminate these foods with pathogenic bacteria. *Bacillus cereus* is one of the common and important food borne pathogens causing spoilage to the foods and producing gastroenteritis to the consumers. This study was carried out to enumerate *B. cereus* and assess their spoilage potentiality and antibiotic resistance pattern.

Methods: Standard plate count was performed using plate count agar while presumptive *B. cereus* was isolated in *Bacillus cereus* agar base supplemented with polymyxin B selective supplement and egg yolk emulsion. Confirmation as *B. cereus* was done by morphological, biochemical and physiological methods. Confirmed isolates were tested for extracellular enzyme production and antibiotic resistance pattern.

Result: Standard plate count exceeded 10^{10} cfu g^{-1} and *B. cereus* count was positive in 57% of the samples. All the 43 *B. cereus* isolates produced amylase and caseinase and were multiple drug resistant. None produced lipase.

Conclusion: Presence of *B. cereus* with spoilage potential and multiple drug resistance require good manufacturing practices (GMPs) and good hygienic practices (GHPs) during food production and storage.

Keywords: *B. Cereus*, Snacks, Sweets, Antibiotic Resistance, Kolkata.

Introduction

With the passage of time various changes in society take place for betterment of daily life. Some of these changes are urbanization, migration to metropolitan cities for better employment opportunity and increase in the number of working couples. But busy working schedule and long working hours leaves little scope for preparation of food daily. According to a recent estimate, the population of Kolkata city is slight more that 5 million while that of Kolkata metropolitan area is around 14.6 million. A considerable number of people of this larger chunk of population residing in suburban and distant areas need to travel regularly to the city. Most of these people depend largely on sweet shops and roadside eateries to satiate their hunger.

Ready-to-eat foods sold in roadside eateries are cheap source of nutrition but occasionally cause numerous public health hazards most importantly gastroenteritis. Over the ages the concept of food vending on streets of Kolkata has changed considerably. Now some vendors can be seen using disposable plates for serving foods, umbrellas for shade, covered container for storing water and even wearing apron to cover their clothes. However, a large number of diverse food items sold by street vendors require different measures to maintain hygienicity. Moreover, these measures are not practiced universally by all food vendors. Scarcity of potable water, shortage of space, irregular cleaning of work place, dust and smoke, insects and rain may contaminate these foods by pathogenic bacteria. Ever increasing temperature and prolonged storage of foods at higher temperature favour growth of these contaminating bacteria. According to WHO more than 80% human diseases are due to contaminated food and water.(1)

Several kinds of snacks are available in the road side eateries to cater a large group of consumers in Kolkata. Some of these foods are prepared elsewhere while others are prepared inside the food stall. The various steps involved in their preparation, disregard for good manufacturing practices (GMPs) and good hygienic practices (GHPs) and inappropriate storage of these foods provide ample scopes of contamination by food pathogenic bacteria. Of the various foodborne pathogenic bacteria *Bacillus cereus* is probably the most underreported one causing frequent outbreak. It is a common and important one due to its sporogenicity and toxigenicity. Endospores are resistant to various stresses and able to survive long under unfavourable conditions. Strains of *B. cereus* have been reported from soil, cereal, spices, vegetables, dairy and meat products, infant foods and dried foods.(2,3) Due to hydrophobic nature, the spores can remain attached to the surfaces of raw ingredients of food, cooking tops, chopping boards and knives even after conventional cleaning of surfaces and subsequently may cause contamination.(4,5) Studies on heat resistance of *B. cereus* endospores have given many decimal reduction values varying with temperature, strains, culture medium and performance. *B. cereus* is not a competitive microorganism. Death of competing

microflora and induction of endospore germination during cooling after heat treatment favours bacterial growth. Toxigenic strains of *B. cereus* may produce two different types of food poisoning: the diarrhoeal type and emetic type. The diarrhoeal type of food poisoning is mostly associated with proteinaceous foods and caused by complex enterotoxins produced during vegetative growth of *B. cereus* in the small intestine.[6] The emetic toxin is mostly associated with strarchy foods produced in foods before consumption.[2,7]

In India, snacks contaminated with *B. cereus* have previously been reported from Mysore city[8] and Srinagar.[9] However, no data on the presence of *B. cereus* on these types of foods in Kolkata is available to the best of our knowledge. Since the requirement and popularity of sweets and snacks among consumers are increasing, this study was undertaken to evaluate the spoilage potentiality and antibiotic resistance pattern of *B. cereus* isolates from laddu, ghugni and soan papdi sold in roadside eateries of Kolkata.

Materials and Methods

Collection of sample: A total of twenty-seven samples comprising of three different types of snack and sweet viz. ghugni, laddu and soan papdi were purchased from roadside eateries in and around Kolkata (capital of West Bengal, a state in India) and stored in sterile container to maintain aseptic condition. Ghugni samples were purchased from sellers in temporary shops on wheeled cart as well as permanent shops while soan papdi and laddu samples were purchased from sweet shops. Samples were then brought to the laboratory within 2h keeping the containers in an ice box.

Measurement of pH: Sample was mixed with carbon dioxide-free distilled water, blended to make a paste and pH measured with a digital pH meter.

Standard plate count (SPC) and presumptive isolation of *B. cereus*: Ten gram of representative food sample was homogenized with 90ml sterile peptone-physiological saline (0.1% w v^{-1} neutral peptone, 0.85% w v^{-1} sodium chloride, pH 7.2) by shaking in an orbital rotary shaker at 150 revolutions per minute for 2 min. Ten-fold Serial dilutions were prepared using the same diluent. 1.0 ml of appropriate dilutions was pour plated in plate count agar while 0.1ml of appropriate dilutions was spread plated on sterile dried plates containing *Bacillus cereus* agar base supplemented with polymyxin B selective supplement and egg yolk emulsion (BCSA). The plates were examined after 72h of incubation at 35° C for standard plate count and 24h of incubation and again after 24h of incubation at room temperature for *B. cereus*. Typical *B. cereus* colonies of bluish-green-blue colour with zones of egg yolk precipitate on the medium (Fig. 1) were counted and calculated as colony forming units (cfu) per gram fresh weight sample. At least three representative colonies were randomly selected from each positive sample and purified by repeated streaking on freshly prepared BCSA plates. Purified colonies were grown on nutrient agar slants and stored at 4°C.

Confirmation of presumptive *B. cereus*: Presumptive isolates were confirmed by morphological, biochemical and physiological characteristics (10). Gram positive, rod shaped, motile, endospore forming cells were tested for acid and gas production from glucose, acetylmethylcarbinol production from MR-VP broth and reduction of nitrate. Cells were confirmed as *B. cereus* when all the tests were positive.

Production of extracellular enzymes: A total of fifty-three confirmed *B. cereus* isolates were tested for the production of enzymes viz. amylase, caseinase, lipase and gelatinase on starch agar, standard method caseinate agar, tributyrin agar base supplemented with 1.0% vv^{-1} tributyrin and nutrient gelatin agar, respectively. Briefly one loopful of 18h old culture grown in nutrient broth was streaked singly on the surface of respective medium (Stab inoculated in nutrient gelatin stabs) and incubated for 72h at 37°C. Amylase production was tested by flooding Lugol's iodine solution on incubated starch agar plates to check formation of clear zones surrounding the colony. Colonies surrounded by clear zones indicated production of caseinase and lipase. Gelatinase production was positive when the stab cultures could not be solidified by keeping the cultures at 4°C for 30 min.

Antibiotic sensitivity pattern: Antibiotic sensitivity pattern of the fifty-three isolates was tested by disc agar diffusion method against twelve antibiotics. Three colonies, grown on tryptone soya agar at 37°C for 24h, were transferred to about 5ml tryptone soya broth (TSB) and incubated at the same temperature for 4-6h until the broth became moderately turbid. A sterile cotton swab was dipped into the inoculum and applied evenly onto Mueller-Hinton agar plate (4mm thick). After drying for 15min, various antibiotic susceptibility test discs were applied aseptically keeping a distance of at least 3cm between their centers. The plates were be incubated at 37°C for 18h. The zones showing complete inhibition were measured and designated as sensitive, intermediate or resistant based on manufacturers zone-size chart.

Results

The results of the pH and microbiological count of the snack and sweets are shown in the Table 1. Minimum pH of 6.4 was of a laddu sample while maximum pH of 7.8 was of both laddu and soan papdi samples. A pH of 7 or more was obtained from seventy-eight percent of the ghugni, seventy percent of the laddu and eighty-eight percent of the soan papdi samples.

Table 1: pH and microbiological count (cfu g^{-1}) of the samples studied

Sample	p^H		SPC		*B. cereus* count	
	Min	Max	Min	Max	Min	Max
Ghugni	6.8	7.5	$9.3x10^9$	$2.51x10^{10}$	$8.4x10^4$	$5.2x10^5$
Laddu	6.4	7.8	$8.3x10^9$	$2.34x10^{10}$	$1x10^3$	$3x10^6$
Soan papdi	6.8	7.8	$6.9x10^9$	$2.09x10^{10}$	$8.8x10^3$	$1.4x10^6$

SPC (Table 1) revealed that microbiological load in the studied samples ranged from a minimum of $6.9x10^9$ cfu g^{-1} in a sample of soan papdi to a maximum of $2.51x10^{10}$ cfu g^{-1} in a sample of ghugni. In terms of average SPC, laddu samples contained the lowest bacterial load ($1.4x10^{10}$ cfu g^{-1}), followed by soan papdi and ghugni samples containing $1.6x10^{10}$ cfu g^{-1} and $1.7x10^{10}$ cfu g^{-1}, respectively.

Table 2: *B. cereus* in samples studied

Parameter	Ghugni	Laddu	Soan papdi	Total
Samples studied	9	10	8	27
Positive sample	5	6	5	16
% positive sample	56	60	63	57

Food wise analysis for the presence of *B. cereus* is shown in Table 2. More than half of the analyzed samples were contaminated with *B. cereus*. In terms of percentage of samples contaminated, soan papdi ranked the top while ghugni samples were least contaminated. Of all the samples, laddu possessed both highest ($3x10^6$ cfu g^{-1}) and lowest ($1x10^3$ cfu g^{-1}) *B. cereus* count. Average *B. cereus* count of the three types of foods considering the positive samples only revealed that soan papdi samples contained highest mean *B. cereus* count of $6x10^5$ cfu g^{-1}. Similarly lowest mean *B. cereus* count of $3x10^5$ cfu g^{-1} was reported from ghugni samples.

Fig. 1: Typical *B. cereus* colony

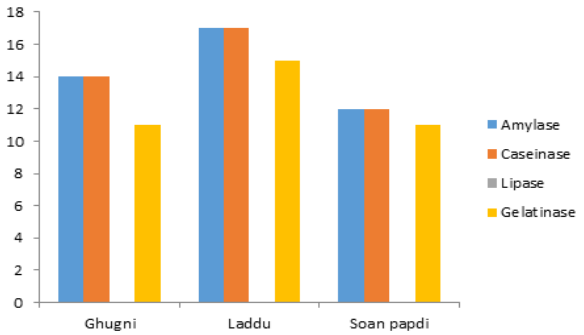

Fig. 2: Number of isolates producing amylase, caseinase, lipase and gelatinase

A total of forty three isolates comprising fourteen from ghugni, seventeen from laddu and twelve from soan papdi could be confirmed as *B. cereus* by morphological, biochemical and physiological confirmatory tests. All these isolates were subjected to the tests for production of extracellular enzymes viz. amylase, caseinase, lipase and gelatinase. Results presented in Fig. 2 reveal that all the isolates produced amylase and caseinase. Altogether eighty six percent isolates were gelatinase positive of which ninety two percent were from soan papdi, eighty eight from laddu and seventy nine from ghugni. None of the isolates produced lipase.

Antibiotic sensitivity (Fig. 3) of the forty three isolates was tested against twelve antibiotics comprising of five inhibiting cell wall synthesis, three inhibiting protein synthesis, two inhibiting nucleic acid synthesis and one each inhibiting cell membrane and folic acid synthesis. All the isolates were multiple-drug-resistant ranging from 4 to 9. Sixty percent of the isolates were resistant to 5 antibiotics. All the isolates were resistant to ampicillin, methicillin and penicillin G. All but one from laddu were resistant to amoxyclav. Ninety-three percent of the isolates except one from ghugni and two from soan papdi were resistant to trimethoprim. On the other hand all the isolates were sensitive to ciprofloxacin and norfloxacin. Sensitivity to chloramphenicol, polymyxin B, erythromycin, vancomycin and tetracycline was for ninety-five, eighty-four, seventy-seven, seventy-four and sixty-three percent of isolates.

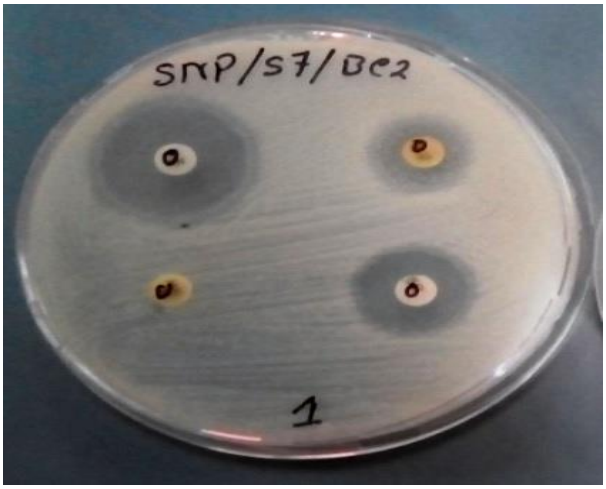

Fig. 3: Plate showing antibiotic resistance pattern

Table 3: Antibiotic sensitivity pattern of the B. *cereus* isolates

Antibiotic (disc^{-1})	Ghugni (n=14)				Laddu (n=17)				Soan papdi (n=12)		
	S	I	R		S	I	R		S	I	R
Cell wall synthesis inhibitor											
Amoxyclav (30µg)			14		1		16				12
Ampicillin (10µg)			14				17				12
Methicillin (5µg)			14				17				12
Penicillin G (10U)			14				17				12
Vancomycin (30µg)	10	1	3		13	3	1		9	3	
Protein synthesis inhibitor											
Chloramphenicol (30µg)	13		1		16	1			12		
Erythromycin (15µg)	10	2	2		13	4			10	2	
Tetracycline (30µg)	9	4	1		13	3	1		5	4	3
Nucleic acid synthesis inhibitor											
Ciprofloxacin (5µg)	14				17				12		
Norfloxacin (10µg)	14				17				12		
Cell membrane synthesis inhibitor											
Polymyxin B (300U)	11		3		16		1		9		3
Folic acid synthesis inhibitor											
Trimethoprim (5µg)	1		13				17		2		10

Discussion

In India, *B. cereus* has been reported from various foods including pulses, rice and rice products, oils, fish, meat, spices, milk and milk products, ice creams.[11-13] But study of this pathogen from foods sold in Kolkata is scanty. The pathogen has been implicated in a number of food poisoning outbreaks throughout the world. So our objective was to ascertain the level of contamination in some popular sweets and snacks having high possibility of *B. cereus*. Ghugni is a popular tangy and spicy street snack made from dried yellow peas garnished with various spices including onion, green chillies and coriander leaves. Laddu is a legume based traditional sweet made by moulding sugar syrup mixed boondi (spherical, crisp, deep fat fried product from Bengal gram flour) into round balls. Soan papdi is a crispy and flaky traditional Indian sweet prepared by processing gram flour, wheat flour, ghee and sugar. The most important basic ingredient of all these foods includes pulses that are predominant source of *B. cereus*. More over preparation procedure, serving and storage of these foods give ample scope of growth of this pathogen.

SPC is a common microbiological test for monitoring microbiological quality of food and feed samples. The result indicates the presence of viable bacterial cells capable of reproduction. Presence of very high SPC in the range of 10^9-10^{10} denotes that the samples were highly contaminated with mesophilic bactereia. Most of the food samples were slightly alkaline in nature. Near neutral to alkaline pH of the samples was congenial for the growth of bacteria in these foods. However, bacterial load in foods depends on other intrinsic factors like water activity, available nutrients, oxidation-reduction potential etc. and various extrinsic

factors including temperature and relative humidity. Detailed study of these factors on the food substrates can give insight into the occurrence and survival of bacteria in these foods.

As all the three types of foods were contaminated with *B. cereus*, consumption of these foods from roadside stalls may cause gastroenteritis. Soan papdi and ghugni samples were the most and the least contaminated foods, respectively, in respect of percent positive samples. Frequent or continuous cooking of ghugni may have killed the pathogens in some samples analyzed. Serving of laddu and soan papdi with same hand that is used to handle money increases chances of exposure with pathogens. Long term storage in ambient temperature favours subsequent growth of the pathogens in laddu and soan papdi samples. Highest mean *B. cereus* count was found in soan papdi. During our collection, ghugni samples were found kept open in a large, flat, round container. The container was heated to keep it warm and spatula was used for serving. However, mixing of unsold ghugni samples of previous days to the fresh products as well as intermittent addition of raw water to maintain required consistency may have contaminated the samples. Both laddu and Soan papdi samples were kept in trays inside three side closed cupboard. These two samples have long shelf life. But inappropriate storage may favour *B. cereus* contamination. *B. cereus* is a known spoilage organism of milk and dairy products causing off flavor, sweet curdling and bitty cream. However, no alteration in apparent organoleptic properties may be observed in non-dairy products posing greater risk to the consumers. All the sixteen positive samples crossed the thresh hold level of 10^4 cfu g^{-1} *B. cereus* count potentiating possible diseases and public health concern. Processing of these types of foods where hands are used for moulding balls of laddu, post-preparative storage, touching soiled notes by bare hands, open air cooking and unhygienic surrounding give ample scope for contamination of these foods by microorganisms. Due to absence of studies with *B. cereus* on these foods, no comparison with previous data is possible. However, contamination of other snacks and street foods by *B. cereus* is reported from India.[8,9,14-17] Growth of mould on the surface of boondi after 7 days of storage at 90% of relative humidity has been reported.[18] Aerobic plate count, total coliform and *Escherichia coli* ranging from 3.21-3.69, 2.42-2.58 and 2.13-2.27 log cfu g^{-1} was reported from ghugni samples sold in the streets of Dhaka city of Bangladesh.[19]

Antibiotic resistance is a worldwide problem attributed to the overuse and misuse of these drugs. In many developing and under developed countries availability of potable drinking water is a challenge but antibiotics are available over the counter without a prescription. Incorrectly prescribed antibiotics in terms of treatment indication, choice of the drug, duration of administration also hasten the complication even in developed countries. Use of subtherapeutic level of antibiotics in livestock management promotes antibiotic resistant bacteria that may again transfer to the consumers through ingestion of foods.[20] Though gastrointestinal disease due to *B. cereus* is generally self-limiting and persisting for around 24-48 h, they may cause severe problem to the young, old and debilitating persons requiring antibiotic therapy. In our study most of the isolates were sensitive to antibiotics inhibiting nucleic acid synthesis and protein synthesis making them possible therapeutic candidates. This result is supported by other workers isolating *B. cereus* from various sources and studying their antibiotic resistance pattern. In a study *B. cereus* was identified from 3.5% of the stool samples collected from acute diarrhoeal patients attending two referral hospitals in Kolkata. All the 54 *B. cereus* isolates were susceptible to amikacin, ciprofloxain, gentamicin, and imipenem. Most of the isolates (>80%) were sensitive to azithromycin and ofloxacin. All the isolates were resistant to amoxyclav and cefixime.[21] In our previous study, *B. cereus* was isolated from 36% of the fresh raw chhana sold in Kolkata. All the isolates were sensitive to ciprofloxacin, levofloxacin and norfloxacin, chloramphenicol and vancomycin. However, all the samples were resistant to amoxyclav, ampicillin, penicillin G, piperacillin/ tazobactam and trimethoprim.[22] In a survey of 39 dried food samples which represented 12 different pulses and cereals 22 (56%) were found to be contaminated with *Bacillus* cereus.[23] *B. cereus* was isolated from 7.4% of stool, 11% of raw milk and 30.85% of meat and meat products.[24] All the 63 isolates were resistant to bacitracin and penicillin G. Ninety and eighty-three percent of the isolates were resistant to carbenicillin and ampicillin, respectively while sixty-five percent of the isolates to amoxicillin and cephalothin. Ninety-five percent of the isolates were sensitive to chloramphenicol. Seventy-five, seventy and fifty-one percent of the isolates were sensitive to neomycin, gentamicin and nalidixic acid, respectively. More than eighty percent of the 35 *B. cereus* isolates from 70 fried rice samples of Malaysia were resistant to streptomycin, ampicillin and tetracycline while more than sixty percent were sensitive to ceftriaxine, chloramphenicol and bacitracin.[25]

From this study it can be concluded that the investigated snacks and sweets sold in road side eateries were highly contaminated with *B. cereus*. As many of these strains may produce diarrhoeal and/or emetic toxin, the foods pose serious public health concern. Quantitatively *B. cereus* count crossed the threshold limit for causing gastroenteritis. Regarding shelf life, production of extracellular enzymes increases spoilage potentiality. Further the isolates were multiple drug resistant showing high degree of resistance against cell wall and folic acid synthesis inhibiting drugs. High degree of suspended particles in the air laden with spores and lack of proper infrastructures require good manufacturing practices (GMPs) and good hygienic practices (GHPs) during their production. Consumers

should be cautious when eating foods from eateries with poor sanitation, insufficient potable water and high level of air pollution.

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