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PREVALENCE OF HEAMOLYSIN-BL AND CYTOTOXIN K TOXINIGENIC *BACILLUS CEREUS* IN MILK/MILK PRODUCTS IN OGHARA AND ENVIRONS

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ABSTRACT
Bacillus cereus; a ubiquitous Gram-positive strain, has been implicated in foodborne diseases and contamination of milk/products as its toxins produce emetic disease and gastroenteritis/diarrheal especially when appropriate hygienic conditions are defiled. In recent times, there is a high interest in the consumption of raw Milk/product in such besmirched state which necessitates study. Current study investigates prevalence of *B. cereus* and detects heat stable (ST) enterotoxin in raw milk/products to ascertain microbiological safety and related health concerns amongst such dairy products. Commercially available raw milk/products were obtained {(with quantity as follows: 5-powdered cowbell milk, 5-Nutri milk, 5-Viju milk, 5-La cream yogurt and 5-fresh Cow/raw milk (Fura da-nunu)} from our local market and public vendors within the Western-Delta-region of Delta State. Samples were subjected to standard microbiological procedures and isolates were characterized by morphological, cultural and biochemical tests attributable to *B. cereus* while the detection of virulence dynamics employed both blood agar and 1-3 days old suckling mice. Amongst the 20 (80%) isolates observed, 14 (70%) isolates were presumptively identified as *B. cereus* with each of them expressing onto the blood agar medium observable β-haemolysis. The ST enterotoxin test shows that among the 14 recovered isolates of *B. cereus*, 50% (7) were positive to heat stable enterotoxin test (ST). Such observation suggests contamination and unhealthy state of such milk/milk products and its consumption poses a potential outbreak hub. There is routine and urgent need for surveillance/re-evaluation as well as microbiological safety of such milk/products within the study environment.

Keywords: *Bacillus cereus;* milk/milk products; heat-stable enterotoxins; appropriate hygiene; suckling mice.

INTRODUCTION

The microbial Genus Bacillus was first described in 1872 with *B. subtilis* as the only specie, however, about fifteen years on, *B. cereus* was added with the general as it was shown to possess multiple metabolic potential in any habitat. Several studies have reported food poisoning attributed to members of Bacillus genus in literature since over ten decade, which also described the isolation of Bacillus species

other than *B. anthracis* from a variety of non-gastrointestinal infections (Shinagawa *et al*., 1996; Igere *et al*., 2021a-c). Since the early 1950's, particularly during recent years, there have been an increasing number of well-documented reports substantiating the relevance of *B. cereus* as food poisoning strain (Kramer et al., 1982). Other accumulating reports have also implicated both *B. subtilis* and *B. licheniformis* as potential food poisoning strains. The prototypical description of its repeated occurrence in association with episodes of food poisoning and/or contamination suggests a potential noteworthy implication of the strain. However, application of *in vitro* entrotoxin-detection techniques applied on strains such as *B. cereus*, *B. subtilis* and *B. licheniformis* in association with gastrointestinal illness have so far failed in affirming the mechanism of pathogenicity of these organisms. Furthermore, some members of *B. brevis* and *B. cereus* have been isolated in large numbers, from contaminated foods in food poisoning outbreaks recently (Kramer *et al*., 1982; Igere *et al*., 2022c-e). *B. cereus* is a psychrotrophic microorganism, aerobic endospore forming strain, potential pathogen of humans and other animals (Logan, 2012), which is associated or implicated with two foodborne illness syndromes: the diarrhoeal illness, promoted by enterotoxins produced inside the host's small intestine, and the emetic illness, promoted by toxins pre-formed in the food (Oh *et al*., 2012; Jeßberger *et al*., 2014). The emetic syndrome starts 1–5 h after the consumption of food contaminated with emetic toxins harboring *B. cereus* and/or preformed emetic toxin cereulide. Its main symptoms are nausea and vomiting while diarrheal syndrome is caused by enterotoxin production in the small intestine by vegetative *B. cereus* cells. As a consequence, this type of disease starts later, 8 - 16 h after consumption of food contaminated with living *B. cereus* cells and/or spores. Other

important related symptoms include abdominal pain and diarrhea. It is important to note that three types of diarrhoea enterotoxins are produced by *B. cereus*: the haemolytic BL toxin (HBL), non- haemolytic enterotoxin (NHE), and the cytotoxin K (CytK) (Ngamwongsatit *et al*., 2008; Ankolekar *et al*., 2009). According to Lindbäck and Granum (2013), about 40% of *B. cereus* strains harbor the hblACD genes responsible for the HBL codification, for this reason the enterotoxins proteins are considered to be the most important toxin of the strains. The presence of *B. cereus* strains that harbor the HBL genes isolated from dairy products has been reported in several studies (Veld *et al*., 2001; Svensson *et al*., 2007; Di Pinto *et al*., 2013; Reis *et al*., 2013; Fernandes *et al*., 2014). *B cereus* has also been reported as contaminant of raw milk and also frequently isolated from a variety of dairy products (Kumari and Sarkar, 2014). Generally, raw milk and dairy products are contaminated by *B. cereus* since it major habitat is the soil and grasses (Igere *et al*., 2020a; O'Connell *et al*., 2013). In addition, some psychrotrophic strains of *B. cereus* are known to grow in dairy at refrigeration temperatures (Montanhini *et al*., 2014), which represents a problem in refrigerated products of milk and other dairy products (Lee *et al*., 2011). In recent time, it has been reported that raw milk, its product as well as dairy food are contaminated by *B. cereus*. Hence, if consumed with such contaminants may lead to a possible outbreak of emesis since milk/its products are regularly consumed by the populace. It is to this end we assess the prevalence of *B. cereus* in milk/milk products within the environments of Western Delta regional district of Delta State. It also sought to identify the enterotoxigenic potential of *B. cereus* isolated from dairy products under controlled incubation temperatures.

MATERIALS AND METHODS

Sample Collection

The samples analyzed were collected from Oghara community and environment.

Batch numbers, expiry dates and the presence or absence of the manufacturers seal was noted. The samples purchased include 5-powdered cowbell milk, 5-nutri milk, 5-viju milk, 5-La cream yogurt and 5-fresh raw/Cow milk (fura da nunu). Sterilization of working bench was achieved by clearing the entire working surface with bleach and 75% methanol solution. Sterility of glass wares, test tubes, media etc, were achieved by autoclave according to manufacturer's instruction.

Bacteriological Analysis

Samples were processed by inoculating fifteen tubes of double strength bijou bottle containing one polymyxin tablet with 10 ml of sample (in each tube), fifteen tubes of double strength growth media with 1 ml (containing durham tube) and fifteen tubes of single strength growth media with 0.5 ml respectively. After incubation at 37°C for 24 hours, the production of gas in any of the Durham tubes was considered positive for gas production.

Isolation of Organism

The incubated liquid media containing samples (as described earlier) were used to inoculate already prepared MacConkey, Blood, Manitol egg yolk polymyxin Agar plates using sterilized wire loop. The cultured plates were then incubated at 37˚C for 48 hours. Biochemical characterization and identification of the test pathogens were carried out using standard identification manual (Cheesbrough, 2005).

Cultural Morphology and Gram Reaction

The microorganism cultured after 48 hours of incubation at 37°C on the MacConkey, Blood agar and Manitol egg yolk and polymyxin agar were Gram stained.

Biochemical Test for Identification of Isolates

Identification of bacteria strains was done using different biochemical tests. These were based on the Gram stain reaction of bacterial strains. The tests include Catalase test, Glucose test, and Manitol test following Cheesbrough (Cheesbrough, 2005).

Confirmatory Test for *B. cereus*

Colonies from Mannitol Yolk Polymyxin agar plates were asceptically subcultured onto nutrient agar slants and Incubated for 24 hours at 37°C and stored for further post Microbiological characterization test. Prepared Gram-stained smears from slants were examined microscopically. *B. cereus* will appear as large Gram-positive bacilli distributed in short-to-long chains; with endospores which are ellipsoidal, central to sub terminal, and do not swell their sporangium.

Labile Cytotoxic test Using Suckling Mice

The aliquot of test organisms were then subcultured onto peptone water and incubated for 24hrs at 37^{o}C. The broth was then centrifuge at 6000rpm for 10 minutes and supernatant collected as toxin extract for each of the isolates that were haemolytic in blood agar plate and confirmed as *B. cereus*. The prepared toxin samples were then divided into two were one of the aliquot was subjected to heat at 100 ^{o}C in a sterile test tube for 30 minutes and the other aliquot remain unheated. 0.1mL of the each extract (Toxin) which was stained with methylene blue was injected into the milk filled stomach of a 4-days old suckling mouse. Each of the mice and treatments/experiments were incubated separately from their mother for 6hrs. The abdomen was dissected and the gut removed and weighed. The weight of the remaining body parts were also taken and the ratio of the weight of gut and weight of body were computed/calculated. A positive heat stable test (ST) is indicated with a ratio value that is greater than or equal to 0.085 or 0.09.

Antimicrobial Susceptibility Test

Mueller- Hinton agar plates were prepared for the isolates. Plates were dried with their lids in a jar (slightly raised) near a lit Bunsen burner. The test organisms from growth on slants were sub-cultured agar plates, purified on nutrient agar plates as innoculum suspension was standardized by 0.5 Mc-Falard standard solution before inoculation onto pre-prepared Mueller-Hinton agar plates using sterile cotton swab (Igere *et al*., 2020b, 2022a-f

Onohuean and Igere, 2022). The selected and specified antibiotics (CLSI, 2015) were centrally placed on the surface of inoculated plates and incubated at 37°C for 24hours. The plates were allowed to initiate growth for about 30 minutes; a multiple antibiotic disks were aseptically transferred directly into the sensitivity plates with the aid of a sterile forceps. Within 30 minutes of application, plates were inverted, incubated at 37°C for 24hrs and then were examined for zone of inhibition around the disk (Selvamohan and Sandhya, 2012).

RESULTS

It was observed from the study that among the twenty-five milk/milk products specimens employed during study, twenty presumptive isolates of *Bacillus* species were recovered from the various milk/products. The identification strategy applied also showed that 20 of the isolates were positive to gas production and glucose (Table 2). Amongst the 20 isolates observed, 14 (70%) isolates were presumptively identified as *B. cereus* with all recovered strains expressing onto the blood agar medium observable β-haemolysis. Other morphological featured and presumptive phenotypic characterization tests applied were also described which presumptively affirmed isolates as *B. cereus*. The ST enterotoxin test shows that among the 14 recovered isolates of *B. cereus*, 50% (7) were positive to heat stable enterotoxin test (ST) (Table 4).

Table 1 The Numbers of *Bacillus cereus* Isolated

Number of specimen analysed	Number of isolates detected	Number of *Bacillus cereus* Isolated
5-specimen La cream yogurt	5	3
5-specimen Powdered Cowbell milk	5	3
5-specimen Cow milk	5	5
5-specimen Viju milk	3	2
5-specimen Nitru milk	2	1
Total of 25 Specimen	20	14

Table2. Results fromPresumptiveand Confirmatory Test

Samples	Gas production	Mannitol (MYP)	Glucose
5-specimen La cream yogurt	5	-	5
5-specimen Powdered Cowbell milk	5	-	5
5-specimen Cow milk	5	-	5
5-specimen Viju milk	3	-	3
5-specimen Nitru milk	2	-	2
Total 25 Specimen	20	-	20

Result of Colonial Morphology, Gram Reaction and Biochemical Tests.

The Table three below shows colonial morphology of tested isolates from various isolation media used during study and the presumptive characterization of strains. It is important to note that the some of the agar plate types used showed strains that were Gram positive bacilli and Gram negative bacilli. Such reports may be associated with the components of the media used which tends to change the morphology and re-shape the retention of the primary stain during Gram staining technique.

Table 3: Result of colonial morphology, Gram reaction and biochemical tests

S/N	Colonial Morpholgy	Gram Reaction	Catalase	Mannitol	Glucose	Presumptive Bacteria Isolated
MCC A	W/PRSOE	GPB	+	-	+	*Bacillus cereus*
B A	W/YRSOE	GPB	+	-	+	*B. cereus*
MS A	WRSOE	GNB	+	-	+	*B. cereus, B. thuringiensis*

Key
Agar, MCC- MacConkey, B- Blood, MS- Mannitol Salt
W/PRSOE- White/Pink Smooth Opaque Entire
W/YRSOE- White/Yellow Smooth Opaque Entire
WRSOE- White Raised Smooth Opaque Entire
GPB- Gram positive Bacillus
GNB- Gram Negative Bacillus

Table 4 The weight of dissected gut and body parts of experimental mice

Numbers of test organism isolated	Weight of body		Weight of gut	
	Unheated toxin	heated toxin	Unheated toxin	heated toxin
3	12.2	11.3	1.11.0	
	11.1	12.8	1.0	1.2
	10.6	11.7	1.0	1.1
3	12.9	11.3	1.2	1.0
	11.1	10.8	1.1	1.0
	10.6	10.7	1.0	1.0
5	12.2	11.6	1.1	
	11.2	10.8	1.0	
	10.6	10.7	1.1	1.0
	10.4	11.3	0.9	1.1
	12.5	11.1	1.1	1.0
2	12.3	12.7	1.1	1.2
	11.7	11.2	1.1	1.0
1	10.2	10.3	0.9	1.0

DISCUSSION

It has been documented that in all cases (either clinical, food poisoning or contamination) of *B. cereus*, the virulence is closely linked with the type of toxin produced (StenforsArnesen *et al*., 2008; Bottone, 2010). Hence the presumptive detection and/or identification of the strains depends on specific microbiological strategies some of which has been applied in this study. The study depicts the isolation and identification of *B cereus* from milk/milk products within Oghara and its environment following specific microbiological strategies as described in the methods section. It was observed that amongst the 25 specimen analysed, 20 of them yielded growth of organisms were 14 (70%) of the isolates from the various specimen were *B. cereus* with the raw cow milk sold in the market having its entire specimen contaminated with *B. cereus* (Table 1). Although, these specimens were stored at refrigeration temperature before the study started, the storage temperature had little or no effect on the specimen as their culture yielded observable growth of colonies. This observation is similar to the report of Van Netten *et al*. (1990) which depicted that some psychrotrophic strains

of *B. cereus* are able to grow and produce enterotoxins up to 4°C, Therefore, temperatures for the storage of milk and milk products had little or no effect on them. It is important to note that the isolated strains showed varying morphological and cultural characteristics which were similar to the control strain used during the study. Furthermore, it was also observed from the study that these organisms (*B. cereus*) isolated produced onto the blood agar culture medium β-haemolysis (14 (70%) of the isolates) which is indicative of strains pathogenic potential on contaminated consumed food as well as expression of haemolysin BL (HBL). This is also similar to the report of Gilbert and Kramer in 1986 that strains of HBL positive *B. cereus* produce haemolysin. In addition, such haemolysis is indicative of the HBL while hemolytic potency varies depending on the species of mammalian blood use for the test. Haemolysis of Sheep erythrocytes does not occur when the organism is incubated with its component alone. Rather, the erythrocytes become sensitized or primed and are rapidly lysed with the addition of some lysing solution. Therefore, hemolysis of erythrocytes in the blood agar plate assay occurs at the point in the diffusion gradient (away from the well) where appropriate concentrations of both B and L exist. Furthermore, the hemolytic activity may causes additional symptoms such as fluid accumulation in the rabbit ileal loop assay, necrosis of villi, submucosal edema, interstitial lymphocytic infiltration, and variable amounts of blood may also be observed in loops that were positive for fluid accumulation (O'Connell *et al*., 2013; Oh *et al*., 2012).

It was also observed from the study that there was no calculated experimental change in the ratio of the weight of whole body of suckling mice and their dissected gut for both the heated toxin sample and the unheated toxin samples, as the ratio range from 0.88 to 1.2. This indicates that the toxin from these isolates (50%) were

thermo stable. It can also be inferred from of the results that *B. cereus* is able to produce some specific enterotoxins which may be emetic. These findings were also documented in the reports of Balaban and Rasooly, (2000); Rajkovic *et al*., (2006); Oh *et al*., (2012). It is therefore worthy of note that *B. cereus* produce spores which are omnipresent, very adhesive and therefore difficult to eradicate from the processing environment. They are highly heat resistant and not easily inactivated by disinfection processes (Drobniewski, 1993). Furthermore, *B. cereus* spores survive heat treatments with relatively low temperatures and are able to persist during low temperature storage. The spores will probably also survive the heating at the preparation of milk/products because only relatively low temperatures are reached and maintained for a limited period of time. This way the spores stay in the food and when the conditions are favorable, these spores are able to germinate and possibly produce enterotoxins (Oh *et al*., 2012). This is inferred in our study as toxins are thernostable. Such heat stable toxins in potential pathogens implicates strains as gastroenteritis/diarrhea causing strains when food/products contaminated by them are consumed as toxins may result/produce emetic disease and diarrheal.

CONCLUSION

It is therefore important to note that the role of *B. cereus* enterotoxins, when formed in food, sources is not clear in food borne diseases. In most cases, the enterotoxins will be degraded during gastro-intestinal passage and it may occur as *de novo* production in small intestine that will cause disease (toxin-mediated infection). On the other hand many authors suggest that preformed toxins under specific conditions may result (or contribute) to symptoms. In contradiction to *B. cereus* enterotoxins, staphylococcal enterotoxins are usually formed in food and are also very stable in terms of heat, pH and enzymes so they may cause food intoxications. One problem of

contamination with *B. cereus* in milk is that it is not eliminated by pasteurization. Another point of attention is the widespread distribution of *B. cereus* and the ability of their spores to survive long-term storage in dried products, as well as thermal resistance of spores which help to explain their spread in wide variety of foods that have been implicated in *B. cereus* foodborne illness outbreaks. Hence one should always bear in mind that *B. cereus* is present everywhere and should take preventative and hygienic measures to prevent growth or cross contamination during food handling. In the light of the above, it is hereby recommended that a routine and regular attention be given by both the government and individuals to the re-evaluation program of food safety and control by exposing these products to Microbiologically test before the sales of such products are ascertain. We are sure that appropriate and adroit adherence to the aforementioned would encourage and reduce to a greater extent microbial contamination of food related products and would greatly impact the safety of these products.

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Conflict of Interest
Authors declared none.

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