

Morphological and microscopical comparison features of *Bacillus cereus* isolates

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Abstract:

Fifty seven samples of milk and infant formula were taken to isolate *Bacillus cereus*. There were 21.55% in milk and 22.5% in infant formula. Polymyxine pyruvate egg yolk mannitol bromothymol blue agar (PEMBA) and blood agar were conducted to grow *B. cereus* in this study. Colony morphology on PEMBA and Blood agar were used along with gram staining and spore staining methods to compare the morphological and microscopically features with the standard strain. The importance of intensity and severity of this organism in food poisoning due to ingestion of incriminated milk and milk products led to this study.

The understanding of the presence and distribution of *B. cereus* discovered the contamination spots in Baghdad markets and the farms in Baghdad districts to make the authorities able to diagnose these contamination areas in order to control and prevent the infection.

The aim of this study is to reveal the availability of *B. cereus* in milk and infant formula in the local dairy farms and the markets of the districts in Baghdad.

الصفات الظاهرية والمجهريّة لعزلات البابلس سيرس

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الخلاصة:

أخذت سبعة وخمسون عينة من الحليب الخام وحليب الأطفال لعزل بكتريا *Bacillus cereus* وجدت هذه البكتريا بنسبة 6.5% و 15% في الحليب الخام بينما كانت النسبة 22.5% في حليب الأطفال. استعمل الوسط الزراعي المتعدد مكسين بايروفيت المحتوي على صفار البيض وسكر التخمر مع البروموثايمول الأزرق ووسط الدم الزراعي لنمو البكتريا وتشخيصها في هذه الدراسة. صنفت بكتريا *B. cereus* حسب أوصافها على الأوساط الزراعية المذكورة أعلاه مع صفاتها لمقارنتها مع

البكتريا القياسية في صبغتي كرام والبوبغ. لأهمية هذه البكتريا في التسمم الغذائي ودرجتها العالية الشدة في الخطورة على الصحة العامة عندما تلوث الحليب ومنتجاته وكذلك لم يتطرق لهذه الدراسة احد في العراق.

إن أهمية معرفة أوصاف هذه البكتريا وأماكن تواجدها في الحيوانات المنتجة للحليب في مناطق بغداد المختلفة وكذلك الأنواع الموجودة من مختلف المناشئء لحليب الأطفال في السوق العراقية يمكن الجهات المسؤولة عن الصحة العامة من مراقبة البؤر لحمايتها من التلوث والسيطرة النوعية على هذه المنتجات. الهدف من الدراسة الكشف عن وجود هذه البكتريا في الحليب و حليب الأطفال في مزارع وأسواق بغداد.

Introduction:

Bacillus cereus is normally available in soil, dust, and water. It can be detected in a variety of raw milk and milk products especially in vegetative form, which exposed directly in contact with the soil (1). This organism produces toxins which caused food borne illness and considered as a significant public health hazard (2). However, the isolation of high levels of *B. cereus* was suggested to be involved in food poisoning (3). *B. cereus* causes gastroenteritis motility and formation of crystalline parasporal inclusion bodies (4). Some investigators estimated 27000 cases per year of food borne diseases from dry milk and other foods in USA only (5).

B. cereus has two different morphology appearances either an endospore or a vegetative cell (6). The vegetative cells of *B. cereus* are facultative aerobic rods, which vary in width from 1.0 to 1.2 μm and in length from 3.0 to 5.0 μm . The rods tend to grow in long chains. The organism is a gram-positive rod primarily characterized by spore formation. During early cell growth,

they are gram-positive, but they can be gram-negative in late growth (7).

The motile *B. cereus* possesses peritrichous flagellae, although nonmotile strains have been observed. The organism was survived in a wide range of temperature from 10-50°C in optimal growth temperatures of 28-35°C. The generation time of this organism occurred between 18 and 27 minutes (8). Growth has been demonstrated over a rather wide range of pH 4.9-9.3. The organism tolerates salt concentrations up to 7.5% while the spores possess a resistance to heat that is typical to other mesophiles, with a germination frequency of up to 100% (9). The spore is characterized by non swelling of the sporangium. Exposure to air does not repress sporulation, and the endospore is resistant to numerous environmental and adverse conditions (10). Extracellular toxins and enzymes of *B. cereus*, including lecithinase, proteases, lactamase, sphingomyelinase, cereolysin, and haemolysin produced food-poisoning (11). This mature protein complex induces

vascular permeability in the skin of rabbits and elicits fluid accumulation in the rabbit ileal loop (12). This component is heat labile (inactivated in 5 min at 56°C) and is sensitive to trypsin and protease (13).

There are two types of food poisoning the short and the long forms have been induced by *B. cereus* (14). Bacterial growth results in production of enterotoxin, and ingestion leads to two types of illness, diarrheal and emetic (vomiting) syndrome. Emetic type of *B. cereus* food poisoning is characterized by short incubation (1-6 hours), nausea, vomiting and abdominal pain. Emetic form resembles *Staphylococcus aureus* food poisoning. *B. cereus* is responsible for a minority of foodborne illnesses (2-5%), causing severe nausea, vomiting and diarrhea. On the other hand, the diarrheal type is associated with a wide-range of foods, has an 8-16.5 hour incubation time and is allied with diarrhea and gastrointestinal pain (15).

The emetic syndrome is caused by a toxin called cereulide that is a dodecadepsipeptide produced by non-ribosomal peptide synthesis (NRPS), which is somewhat unusual in itself, 'poisoning in its symptoms and incubation period. The emetic toxin is different from the diarrheal-type toxin in function and has a molecular weight of about 1.2 kDa (16,17). This toxin has been determined to be a cereulide, an ionosphoric water-soluble peptide

that is closely related to the peptide antibiotic valinomycin. It induces the formation of vacuoles in Hep-2 cells and is highly resistant to heat; its activity is not lost after heating at 120°C for 1 hour. Additionally, this cereulide is stable from pH 2.0-11.0, is not hemolytic, and does not induce vascular permeability in the skin of the rabbit (18).

The diarrheagenic toxin is an extracellular protein complex that elicits diarrhea in monkeys and can be identified in culture fluids by serological assay (19). A number of the properties of the diarrheal and emetic toxins produced by *B. cereus* were described (20). This complex exhibits a number of phenomena, including haemolysis, cytolysis, demonecrosis, vascular permeability, and enterotoxic activity, while no single enterotoxic has been demonstrated, the HBL component appears to be responsible for the diarrheagenic syndrome (21).

The aims of this study are to discover the contamination areas in Baghdad with *B. cereus* as well as to understand the type of *B. cereus* strains available in these areas.

Materials and Methods:

Bacillus cereus (ATCC 11778) was obtained from the Culture Collection, Department of Microbiology, University of RMIT (Australia) used as a positive control to compare our results with this standard organism.

The cultures were kept in nutrient agar slope for maintenance. Peptone water was used to avoid the strains variation and keep the strain a live as long as possible.

Collection of milk samples were conducted in sterile bottles from different areas in Baghdad during winter and spring seasons in 2009. The samples were transported into the laboratory in iced boxes to avoid multiplication of microorganisms during transport time (around 2 hours). The samples were transferred in peptone water (1ml milk to 9 ml peptone water) then 0.1 ml was streaked on the surface of PEMBA agar (already have been prepared) spread by sterile glass rod. The PEMBA plates were incubated in the incubator at 37°C overnight. Then the plates were incubated further at room temperature for one more day. Gram stain and spore stain as well as colony morphology were conducted to suspected bacteria. Blood agar was used to identify sheep RBCs hemolysis. The colonies were streaked on nutrient agar slope to keep the cultures until further testing.

B. cereus media 40g/L and Supplement (Oxoid) 5ml /L with Polymyxine 1 ml/ 100,000 I.U./ ml were supplied by Central Health Laboratories in Baghdad, egg yolk was purchased from Oxoid Supplier Representative in Baghdad. Blood agar was prepared from nutrient agar plus 8-10% sheep blood. Peptone water

(Oxoid) 0.1% was prepared and used as a diluent. Peptone water (Lab M) was prepared as manufacture instructions. Sheep blood from the College of Veterinary Medicine Farm. The media were used in isolation of *B. cereus* is polymyxine pyruvate egg yolk mannitol bromothymol blue agar (22). Weigh out 20.5g of PEMBA into 1L screw bottle add 500 ml distilled water mix very well, autoclave 121°C for 15 minutes, cool down to 50 °C then add aseptically 25 ml egg yolk (Oxoid) and polymyxine supplement (50,000 I.U. polymyxine), pour 15 ml into petri dishes. PEMBA plates were kept in refrigerator until use, shelf life is one month.

Peptone water 0.1 % was prepared in I L size flask then dispense in 90 ml aliquots as follows: One gram of peptone water (Lab M) was dissolved in a liter of distilled water mixed well then was dispensed 90 ml into 100 ml size screw bottle, the pH of peptone water was adjusted to be 7.0, then autoclaved at 121°C for 15 minutes. Peptone water cooled down and kept them in the refrigerator until used.

To homogenize the milk samples a mixer was used in case of raw milk. In case of infant formula was weighed 10 g of infant formula added to 90 ml sterile 0.1% peptone water mixed the samples by stomacher and mixer to homogenize the samples. Apply 0.2 ml of milk samples or infant

formula samples on PEMBA agar and incubated overnight in the incubator at 37°C. The suspected colonies were picked from the PEMBA plates to confirm them by colony morphology on blood agar, gram stain and spore stain. Positive colonies were subcultured on blood agar to check the hemolysis characterization. For further confirmation positive cultures were kept on nutrient agar slopes.

Results:

Out of the total number of samples collected from different areas and several farms in Baghdad were fifty seven samples. There were six positive samples out of the fifty seven samples 10.5% total number of samples have been tested (**Table 1**).

One out of sixteen samples 6.25% collected from the farm of College of Veterinary Medicine, University of Baghdad was positive. Three out of twenty samples 15% have been tested from Alfdhailia farm gave positive results. Two out of nine samples 22.2% were taken from different brands and sources of infant formula

were positive (**Table 2**).

The colony morphology of *B.cereus* compared to the standard colony morphology to identify the organism. *B. cereus* colony characterized in PEMBA medium by blue, dull, crenate, flat, irregular, undulated margin, large size 5 mm (**White arrow Figure 1 A**) and was surrounded by a zone of precipitation look like as an extracellular products at the edge of the colony (**Black arrow, Figure 1 B**).

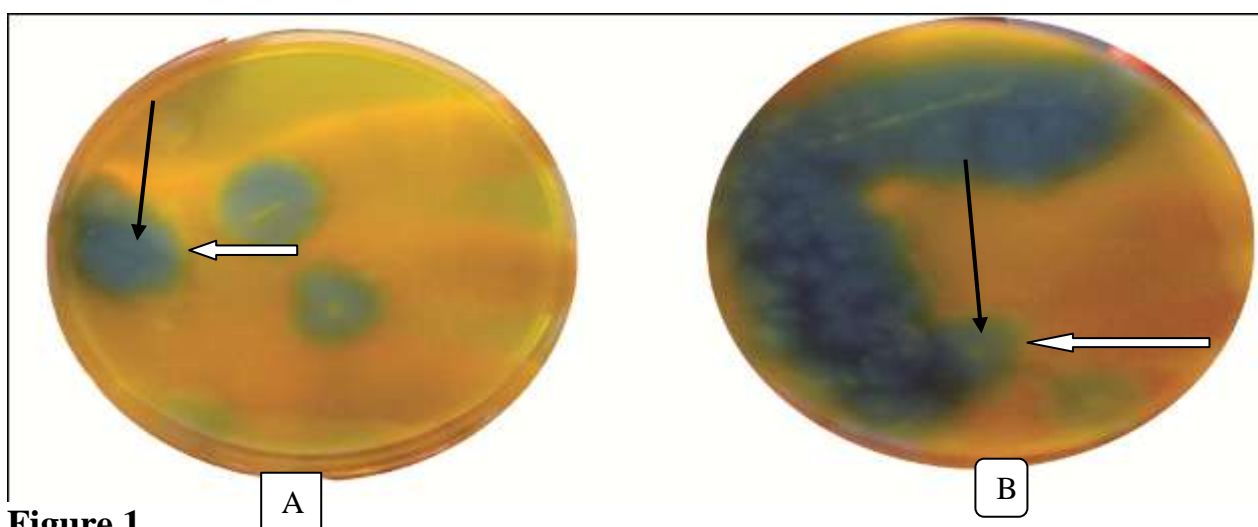
The standard *B. cereus* colony characterized in PEMBA medium by blue, dull, crenate, flat, irregular, undulated margin, large size 5 mm (**White arrow Figure 2A**) and was surrounded by a zone of precipitation colony (**Black arrow Figure 2B**).

B. cereus colony on blood agar (**Figure 2A**). The hemolysis of *B. cereus* was showed as an extracellular area of the colony (**Figure 2B white arrow**).

Gram stain of *B. cereus* determined the long and width as well as the colour of the organism, blue for gram positive (Figure 3, white arrows) and red for gram negative (Figure3, yellow arrow)

Table 1. Comparison between Local Isolates and Standard *B. cereus*

Characteristics	<i>B. cereus</i> Isolates (n=6)	Standard <i>B. cereus</i>
Colony morphology on PEMBA	Blue, Dull, Crenate, Flat, Irregular, Undulated Margin, Large size 3-4mm and surrounded by a zone of precipitation. Clear zone on the border of precipitation	Blue, Dull, Crenate, Flat Irregular, Undulated Margin, Large size 5mm and surrounded by a zone of precipitation.
Blood Agar	Hemolysis	Hemolysis
Gram stain	Gram positive, rods 4µm to 5µm long and 1µm to 1.5µm wide	Gram positive, rods 4µm to 5µm long and 1µm to 1.5µm wide
Spore stain	Spore location in the centre of cell, not swell the sporangium.	Spore location in the centre of cell, not swell the sporangium.

**Figure 1.**

A. *B. cereus* colony characterized in PEMBA medium by blue, dull, crenate, flat, irregular, undulated margin, large size 5 mm (**White arrow**).

B. and was surrounded by a zone of precipitation look like as an extracellular products at the edge of the colony (**Black arrow, Figure 1 B**).



Figure 2. A. The hemolysis of *B. cereus* (white arrow)
B. Extracellular area of the colony (black arrow).

Table 2. Isolation of *Bacellus cereus* Organisms

Area		Samples	Positive	%
Milk from Farm of College of Veterinary Medicine/Al Ameria		16	1	6.25
Milk from Seven Nissan Farm		12	0	0
Milk from Al Fdhalia Farm		20	3	15
Infant formula	Five samples of bulk quantity, Sudanease origin	5	2	40
	Four samples of tin packed an Indian origin	4	0	0
Total		57	6	10.5

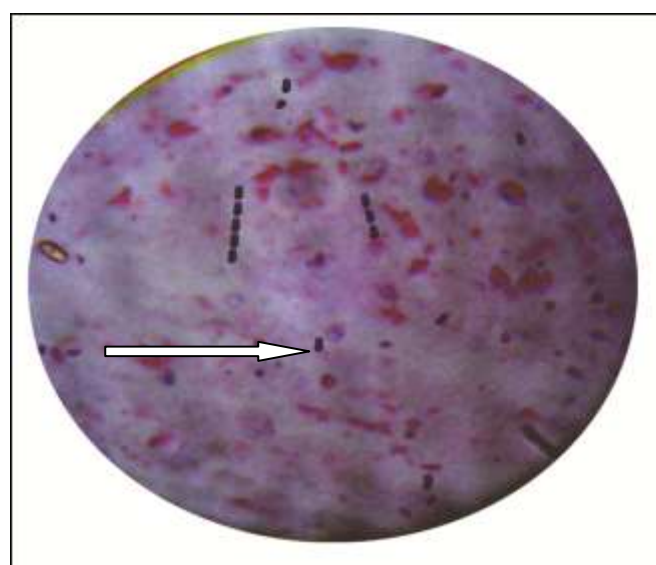


Figure 3. Gram stain of the *B. cereus* white arrow (- equal 3 Micron)

Discussion:

Phenotypically, the standard *B. cereus* and local isolates were similar in colony morphology characterization and microscopic features (**Table 1**). Blue color in PEMBA plates indicated that the basic medium of mannitol was not ferment by *B. cereus* as well as egg-yolk precipitate surrounding the colonies were noticed in both strains (**Figure 2**). These results agreed with the results recorded by (23), the authors were investigated the presence of lecithinase (phospholipase Q and the absence of mannitol fermentation in *B. cereus* produced blue color colonies and showed the precipitation of egg yolk. Presumptive colonies were surrounded by an egg-yolk precipitate and have a violet-red background on MYP and a turquoise to peacock blue color on PEMBA. Both media contain polymyxin to inhibit the growth of competitive organisms.

The hemolysis indicated that the strains isolated from milk and infant formula in this research could be a virulent strain. Our results agreed with (24) who indicated that the hemolysin was a spore-forming toxin of the opportunistic pathogen *B. cereus* thought to be related to the virulence of the organism.

There was a precipitating zone on the edges of the *B. cereus* colonies dissolving the protein materials by specific enzyme called proteinase (Figure 2 small

arrow). These results were in accordance with (25) who believed that the zone surrounded the colonies in PEMBA agar was due to proteolysis enzymes produce by *B. cereus*. There were assumed to be proteins substances which act as antibacterial products by the milk strain of *B. cereus*. Furthermore, these substances inhibit other bacterial strains. These results were reported by (26) who established to produce proteinaceous substances which inhibited the growth of other *B. cereus* isolates.

B. cereus is normally gram positive rod. Sometimes it is a variable due to the integration of the cell wall with the crystal violet, the exposure duration to alcohol in the process of decolonization during the gram staining method and the old of the culture used for the staining. Usually the gram stain of the organism appears blue in color (Gram positive). Sometimes the gram stain colored *B. cereus* as a red (Gram negative) due to the factors which mentioned in the gram stain process.

The enzymatic products released by *B. cereus* isolates from raw milk and milk products could be participated in the food borne poisoning. Both emetic and diarrheal forms of foodborne illness were associated with the enzymatic substances produced by *B. cereus*. The relationship between pathogenicity of *B. cereus* and its

enzymes were linked in the epithelial cell adhesion and *B. cereus* spore (27).

In conclusion, *B. cereus* was identified in milk and different infant formula in Baghdad districts and markets. This study is essential for further studies to identify the epidemiology and pathogenicity in this country.

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