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INCIDENCE AND CHARACTERIZATION OF *BACILLUS CEREUS* ISOLATED FROM EGYPTIAN FOODS.

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وجود باسيلس سيرس في الأطعمة المصرية وتوصيفه

يسرى السيد صالح و محيي الدين الفولي
ماري صبحي خليل و مرفت أبو شتيت

عزلت ١٧٨ سلاسة لميكروب باسيلس سيرس من أطعمة مصرية مختلفة وقد استخدم لهذا الغرض نوعان من البيئات الخاصة وعرفت السلالات تبعاً لصفاتها المورفولوجية والبيوكيميائية باستخدام مفاتيح وطرق التعريف المختلفة .

وجد أن جميع الأطعمة المستخدمة في الدراسة كانت ملوثة بميكروب باسيلس سيرس بالنسب الآتية : اللحم المفروم ٩٦٪ والسجق ٦٠٪ وحبوب الارز ٤٨٪ وكلا من الكشري والآيس كريم ٤٤٪ واللبن المبستر ٣٦٪ .

وقد درست قدرة باسيلس سيرس المعزولة من الناحية البيوكيميائية ، وتأثيره المرض والسام على فئران التجارب وكذلك تكسير كرات الدم الحمراء وانتاج انزيم الفوسفوليبيز وكذلك حساسيتها للمضادات الحيوية . وقد وجد أن بكتريا باسيلس سيرس تقاوم البوليمكسين والبنسيلين والكربنيسيللين والكلورامفينيكول والتراي ميثوبريم والسلفا ميثوكزازول .

Key Words: Bacillus cereus, food contamination, pathogenicity, toxigenicity.

ABSTRACT

In this study, 178 strains of *Bacillus cereus* were isolated from different Egyptian foods. Isolation was performed, using MYP and PEMBA media and confirmed by staining and biochemical tests. *B. cereus* occurred in 98% of test minced meat, 60% of sausage, 48% of rice grains, 44% of Koshari or ice-cream and 36% of pasteurized milk samples.

The characteristics of these *B. cereus* isolates in terms of biochemical reactions, antibiotic susceptibility, pathogenicity and toxigenicity were studied. *B. cereus* was especially resistant to polymyxin, penicillin, carbencillin, chloramphenicol and trimethoprim/sulphamethoxazole.

INTRODUCTION

The role of *Bacillus cereus* in outbreaks of food-borne illness is becoming well documented. *Bacillus cereus* is a widely distributed bacterium, it has been isolated from rice, spices, meat, eggs, and dairy products (Johnson *et al.*, 1984). It is recognized as causing diarrhoeal and emetic types of food-poisoning outbreaks. As reported in the U.S.A. by Ahmed *et al.*, (1983), 9% of the raw milk, 35% of the pasteurized milk, 14% of the cheese and 48% of the ice-cream samples were contaminated with *B. cereus* . Conversely, no fermented milk was found contaminated. Shinagawa *et al.* (1988) enumerated microbial populations in meat product additives such as spices, proteins, starch, salts, sugar and colours. Multiplication of *B. cereus* in dairy products is not only of concern as public health hazard, but also as a cause of economic losses through spoilage of contaminated products. To estimate the role of a microorganism as a food contaminant and pathogen in products, one must know the incidence, growth, germination and toxin production by that microorganism in these products.

The present study was undertaken to asses the frequency and ratio of *B. cereus* contamination in selected food products purchasable at the Egyptian local markets. In addition, current methods used for isolation and identification of *B. cereus* from food products are evaluated.

MATERIALS AND METHODS

All food samples were collected from different street vendors, shops, super-markets and groceries of various districts of Cairo, Egypt. Twenty five samples were taken from each of Koshari, pasteurized milk, ice-cream, dry rice grains, minced meat and sausage bags weighing 400-500 gm related to five different factories.

The food sample was diluted or ground with sterile saline solution (1: 100 v/v or w/v). One ml of this suspension was transferred to the surface of plates with either one of two different media, namely the *B. cereus* agar (PEMBA) reported by Holbrook and Anderson (1980)

and *Bacillus* selective agar (MYP) medium of Mossel *et al.* (1967). Colonies showing the typical characteristics were purified by several consecutive streaking on the same specific media, then transferred to blood agar medium (Cowan and Steel, 1974) and nutrient agar medium (Claus and Berkeley, 1986).

Identification of the isolates was based on the biological and biochemical characteristics according to the International Identification Keys (Cowan and Steel, 1974; Geopfert, 1976; a) A Colour Atlas of *Bacillus* Species, 1983 and b) Bergey's Manual for Systematic Bacteriology, 1986).

The biochemical confirmatory tests of bacterial isolates were: starch hydrolysis, acetylmethyl carbinol production (Voges-Proskauer reaction), nitrate reduction, glucose, mannitol and xylose fermentation were determined by the methods described by Smith *et al.* (1952). Gelatin liquefaction was performed at 30°C for four weeks. Liquefaction was tested at 3-day intervals after keeping the tubes at 4° C for 30 minutes, failure to solidify indicated gelatin hydrolysis. Growth on phenethyl alcohol agar. was determined by the method of Knisely (1965). Growth on nutrient agar containing 7% sodium chloride, at 30° C, for 3 weeks was daily observed. Catalase activity was tested by smearing the culture on a slide with a drop of 10% hydrogen peroxide. Oxygen bubbles evolution indicated positive reaction. Egg-yolk reaction was determined by inoculating the basal medium followed by incubation at 30° C, for 3 days. Appearance of a heavy white precipitate in or on the surface of the medium indicated a positive result.

Sensitivity test was demonstrated by using the P Y agar medium, described by Bernhard *et al.* (1978). Pathogenicity was tested according to the method of Norris and Wolf (1961). Toxicity to mice, haemolysin and phospholipase activity were determined according to Parry *et a.* (1983). The haemolytic activity was detected by using the medium described by Fossum (1963), and phospholipase activity using the medium described by Turnbull and Kramer (1983).

RESULTS AND DISCUSSION

Fig. (1) shows that the twenty five tested samples of each food were contaminated with *Bacillus cereus* in a ratio of 96% for minced meat, 60% for sausage, 48% for rice grains, 44% for Koshari or ice-cream and 36% for pasteurised milk. Colonies on PEMBA and MYP media were typical of *B. cereus;* they were crenated. flat, dry, about 5 mm in diameter and surrounded by zones of precipitate [PLATE 1, 2]. The colonies were greyish-white, opaque, granulated surface with a ground-glass appearance surrounded by haemolytic zones [PLATE 3]. These colonies were always readily countable after 24 hours incubation at 30°C.

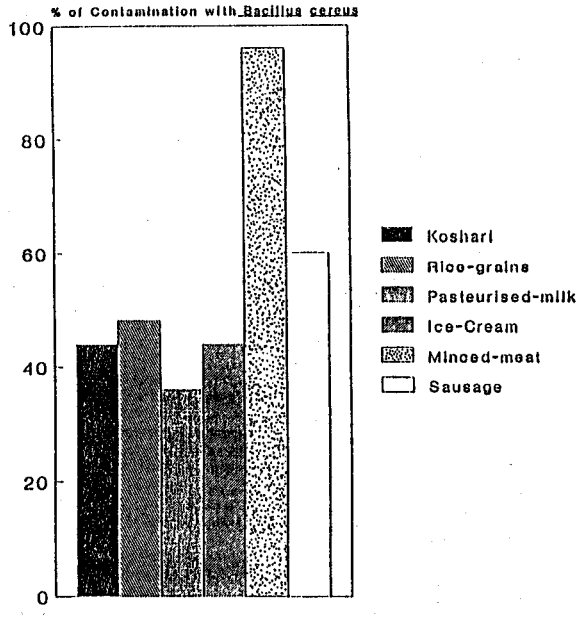

Fig. 1: Percentage of incidence of *Bacillus cereus* in different foods.

The morphological characters were checked by Gram and spore stains. The isolates were subjected to physiological and biochemical tests. The data in Table (1) reveal that all 178 isolates could liquefy gelatin, are catalase-positive, produce precipitate from egg-yolk, reduce nitrate and produce acid from glucose. A total of 165 of these isolates showed positive blood haemolysis, 132 could hydrolyse starch, 170 produced acetylmethyl crabazole (positive Voges-Proskauer reaction) and 175 could grow on 7% sodium chloride agar medium.

Table 1
Biochemical reactions of *Bacillus cereus*

Biochemical test	Number of Strains		Percent of +ve Strains
	Positive	Negative	
Blood Haemolysis	165	13	92.69%
Starch Hydrolysis	132	46	82.02%
Gelatine Liquefaction	178	---	100.0%
V-P Test	170	8	95.50%
Catalase test	178	---	100.0%
Egg-yolk reaction	178	---	100.0%
Nitrate Reduction	178	---	100.0%
Growth on 7% NaCl	175	3	98.31%
Acid Glucose	178	---	100.0%
Production Mannitol	4	174	2.24%
From Xylose	4	174	2.24%

The pathogenicity and toxin activity were tested using one selected strain from each of the tested food materials. Tables (2 & 3) show that the strain *B. cereus* $K_{11}s$ was the most active since it caused 100% death of infected mice, and gave positive results with all toxigenic tests [PLATE 4]. Therefore, this strain was chosen and a study of the different factors controlling its growth was conducted.

Table 2
Pathogenicity of the selected strains of *Bacillus cereus*

Tested Strains	Number of Mice		% Dead
	Inoculated	Dead	
Standard Strain	5	1	20%
$M_{17}M$	5	3	60%
$K_{11}S$	5	5	100%
$L_{24}M$	5	3	60%
S_9S	5	2	40%
$I_{25}S$	5	1	20%
$R_{16}M$	5	3	60%

$M_{17}M$: specimen of milk
$K_{11}S$: specimen of koshari
$L_{24}M$: specimen of minced meat
S_9S : specimen of sausage
$I_{25}S$: specimen of ice-cream
$R_{16}M$: specimen of rice

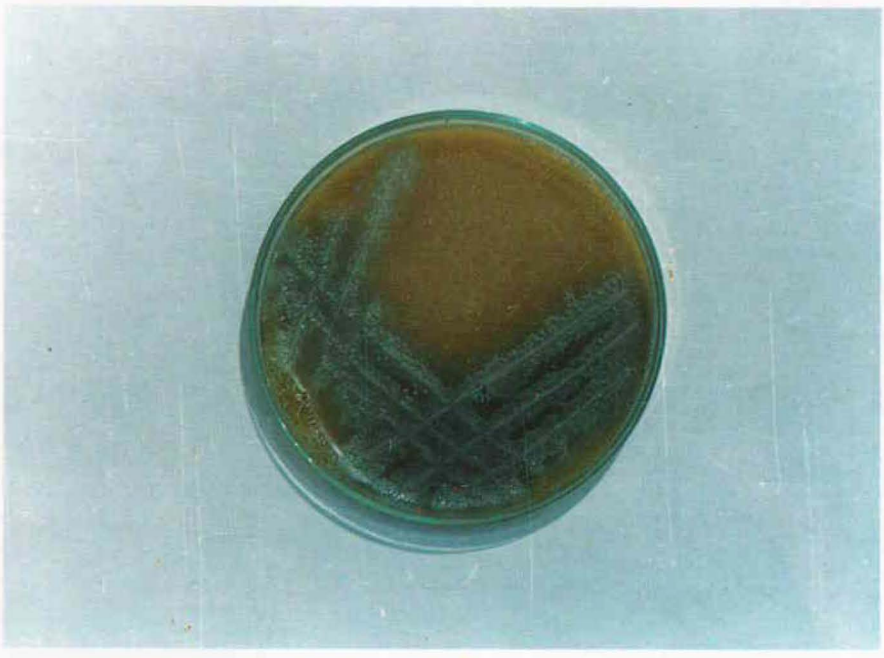

PLATE 1: *Bacillus cereus* colonies on selective agar medium (PEMBA).

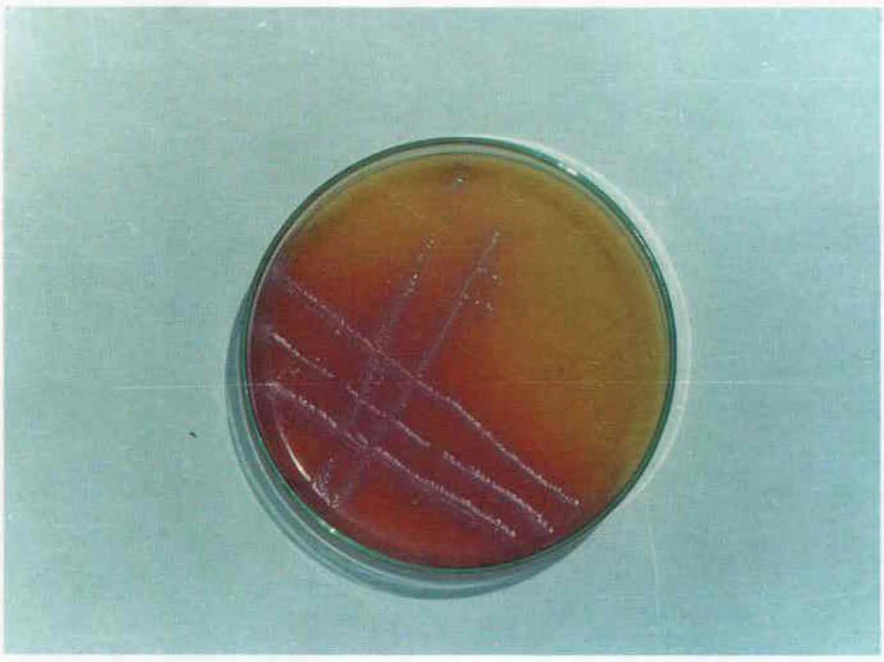

PLATE 2: *Bacillus cereus* colonies on Mossel selective agar medium (MYP).

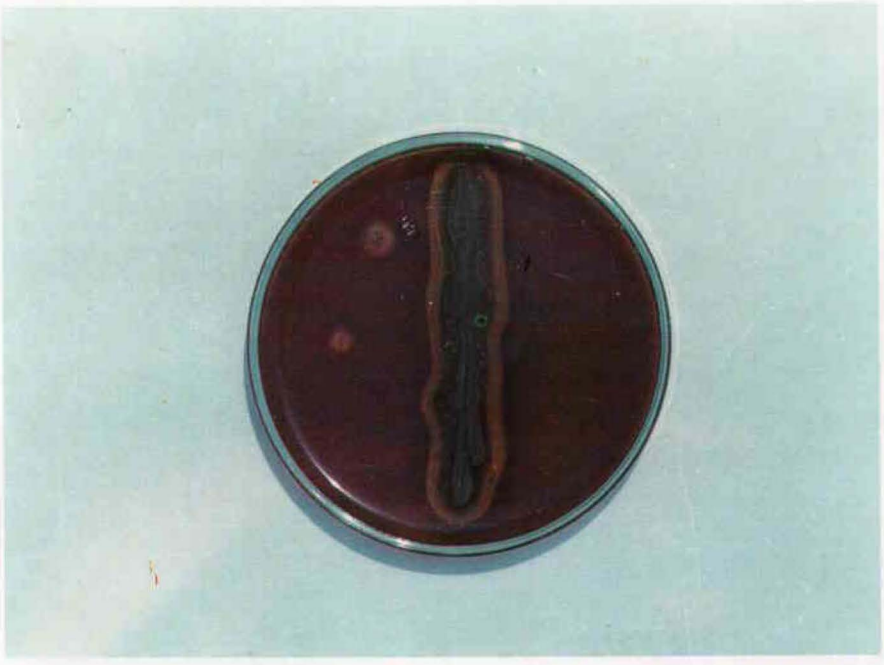

PLATE 3: Effect of *Bacillus cereus* on sheep blood agar.

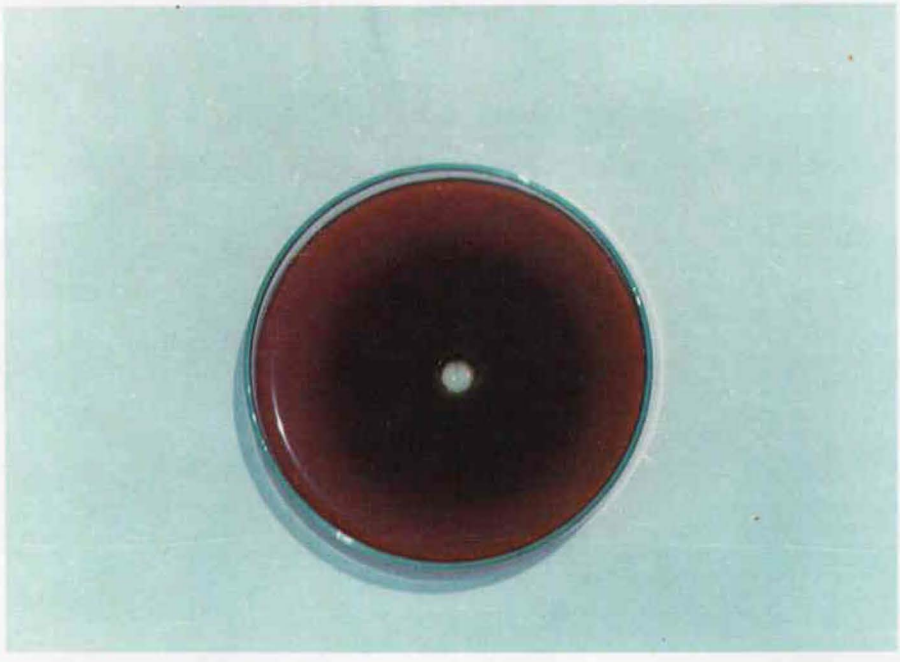

PLATE 4: Effect of *Bacillus cereus* $K_{11}s$ toxin on blood agar after 18 hours incubation.

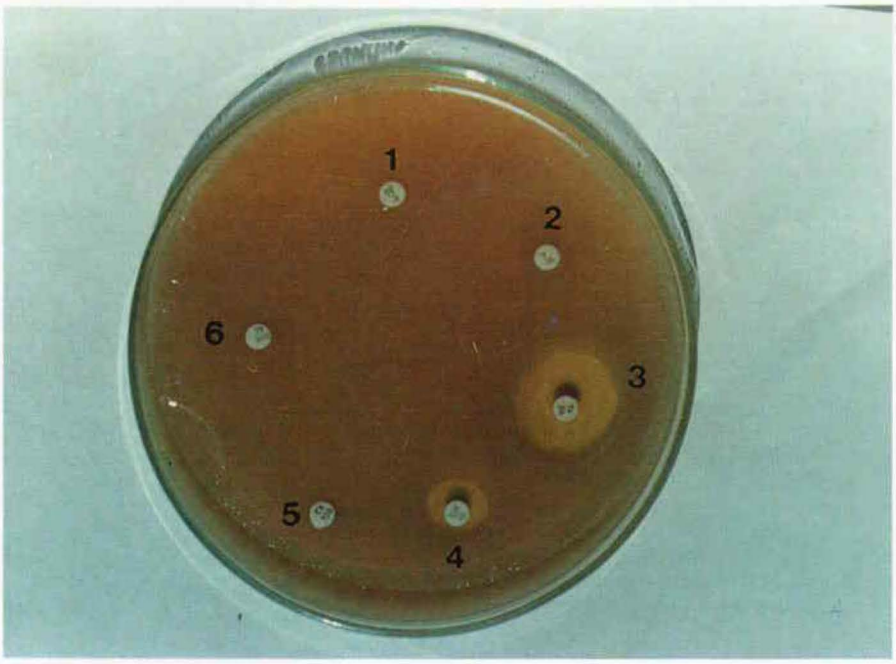

PLATE 5: Sensitivity test of *Bacillus cereus* $K_{11}S$.
1: Chloramphenicol (30 µg), 2: Penicillin (10 µg), 3: Tetracycline (30 µg), 4: Gentamycin (10 µg), 5: Carbenicillin (100 µg), 6: Polymyxin (300 µg)

Table 3
Toxigenicity of the selected strains of *Bacillus cereus*

Tested Strain	Mouse Lethal Toxin	Haemo-lysin	Phospho-lipase
Standard Strains	+ +	+	+
$M_{17}M$	+ +	+	+
$K_{11}S$	+ +	+	+
$L_{24}M$	- -	+	+
S_9S	+ +	+	+
$I_{25}S$	+ +	+	+
$R_{16}M$	+ +	+	+

+ + Lethal effect
+ Haemolytic effect and phospholipase production
- Negative result

The susceptibility of *B. cereus* $K_{11}S$ to different antibiotics is recorded in Table 4 and PLATE 5. The results indicate that this bacterium was absolutely resistant to penicillin, carbenicillin, polymyxin, chloramphenicol, trimethoprim and sulphamethoxazole, as reported by Finegold and Baron (1986). This strain was highly susceptible to tobramycin, amikacin, norfloxan; moderately susceptible to gentamycin, oxolinic acid and less susceptible to keflex.

Table 4
Antibiotic susceptibility of *Bacillus cereus* $K_{11}S$.
(mm diameter of inhibition zone)

Antibiotic	Disc	Response	Diameter
Tobramycin	30 µg	+ + + +	34
Erythromycin	15 µg	+ + + +	30
Rifampicin	30 µg	+ + + +	30
Tetracycline	30 µg	+ + + +	30
Amikacin	30 µg	+ + + +	34
Norfloxacin	10 µg	+ + + +	26
Gentamicin	10 µg	+ + +	16
Oxolinic acid	30 µg	+ + +	22
Keflex	30 µg	+ +	24
Polymyxin B	300 U	- ve	
Carbenicillin	100 µg	- ve	
Penicillin	10 U	- ve	
Chloramphenicol	30 µg	- ve	
Trimethoprim	1.25 µg	- ve	
Sulphamethoxazole	23.75 µg		

+ + + + Highly susceptible
+ + + Moderately susceptible
+ + Less susceptible
-ve Resistant

The positive contamination of these food samples is in good agreement with the reports of Mossel *et al.* (1967) and Nygren (1962). Lotfi *et al.* (1988) reported that 75% of raw minced meat was contaminated with *B. cereus* . The high incidence percentage

may be attributed to the abundance of amino acids, vitamins and essential nutrients in the meat. Fresh meats of beef, pork, lamb as well as fresh poultry and sea foods, have pH values within the growth range of most of the organisms (Jay, 1986). That *B. cereus* is less frequent in sausage might be attributed to the presence of curing agents and the low pH of the product. Silliker *et al.,* (1980) mentioned that lowering the pH of meat emulsions, by adding acidulants decreased the likelihood of growth of spoilage- and disease-producing bacteria. This result was confirmed by Wong and Chen (1988) who added that acetate, formate and lactate (at 0.1 M) completely arrested multiplication of *B. cereus* at pH 6.1, 6.0 and 5.6, respectively.

Using pasteurised milk in making ice-cream favoured the domination of *B. cereus* over that was in milk itself (36%). The percentage increased in ice-cream to 44% contamination, while Wong *et al.* (1988) reported 52% and Ahmed *et al.* (1983) reported 48% contamination. *B. cereus* may be present in higher proportions in the raw materials, or may have a considerably higher growth rate during the manufacturing process in summer, due to the naturally higher temperature of this season than the others. In addition, the display cabinets for ice-cream or its machines, may be more contaminated by *B. cereus* in summer.

Few papers reported on the drug resistance of *B. cereus* (Bernhard *et al.,* 1978; Johnson *et al.,* 1984; Chung and Sun, 1986). It is highly susceptible to nisin, aureomycin, dehydrostreptomycin, terramycin, bacitracin, oxytetracycline, chloramphenicol and gentamycin, but slightly susceptible to neuromycin, cloxacillin, ampicillin and penicillin (Johnson, *et al.,* 1984).

The incidence of contamination of food products by *B. cereus* in Egypt is fairly high. Our isolates showed haemolysin and cytotoxin activities which may suggest that *B. cereus* toxin was associated with the presence of plasmid (Bernhard *et al.,* 1978; De Buono *et al.,* !988). Haemolysin, mouse-lethal toxin and phospholipase activity assay methods were valid for examining the toxigenicity of *B. cereus*. Accordingly, it is clear that neither the risk of food poisoning caused by *B. cereus* nor the disregard of this organism as a causal organism of food poisoning should be neglected.

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