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INCIDENCE AND ANTIBIOTIC RESISTANCE OF PSYCHROTOLERANT *BACILLUS CEREUS* GROUP IN ICE CREAM SAMPLES, WITH SPECIAL REFERENCE TO *BACILLUS THURINGIENSIS*

(With 5 Tables)

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مدى تواجد مجموعة الباسيلاس سيريس المقاومة للبرودة ومقاومتهم لمضادات البكتيريا فى عينات الآيس كريم مع اشارة خاصة للباسيلاس ثورينجينسيس

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تسبب بكتيريا باسيلاس سيريس تسمم غذائى بواسطة السموم المعوية. ولذلك تم البحث عن مجموعة ميكروب هذه الباسيلات المقاومة للبرودة فى ١٥٠ عينة آيس كريم. استخدم مستنبت الاجار المحتوى على مانيتول – صفار البيض والبوليميكسين ب كمستنبت انتقائى لهذه المجموعة. ثم تم التعرف على كل ميكروب بالتجارب البيوكيميائية وعلى هذا وجدت هذه المجموعة بنسبة ٣٢٪ من العينات الكلية للآيس كريم المفحوصة. كما تم عد تواجد الميكروب فى كل جم من كل عينة. وقد وجد باسيلاس ثورينجينسيس فى الثلاث انواع من الآيس كريم. كما تم تحديد مقاومة مجموعة باسيلاس سيريس لثمانية أنواع من المضادات الحيوية بطريقة انتشار الأقراص. ووجدت معظم العزلات مقاومة للمضادات الحيوية الآتية: الأمبسيلين ، الأموكسيسيلين ، الستريبتومايسين والنيومايسين، بينما وجدت معظم العزلات حساسة لكل من: الأريثرومايسين ، الكلورامفينيكول ، سيفالاكسين والكانامايسين. ولهذا فوجود الميكروبات المقاومة للمضادات الحيوية توضح قلة وضعف الظروف الصحية خلال التصنيع وهو يسبب خطورة لصحة المستهلك.

SUMMARY

Bacillus cereus causes food-poisoning by means of enterotoxins with either emetic or diarrheal effects. Hence, psychrotolerant *Bacillus cereus* group occurrence in 150 ice cream samples was investigated. Mannitol-

egg yolk-polymyxin B (MYP) agar medium was used as selective medium for isolation of this group. All isolates were identified by several biochemical tests. Accordingly, psychrotolerant *B. cereus* group was found in 32% of the total ice cream samples. Also, psychrotolerant *B. cereus* group count in each sample was estimated. *B. thuringiensis* was isolated from the examined three kinds of ice cream samples. Antibiotic sensitivity test was done by disc diffusion method using 8 different antibiotics. High resistance rate was found to ampicillin, amoxicillin, streptomycin and neomycin. Whereas, sensitive to erythromycin, chloramphenical, cephalexin and kanamycin. Therefore, the presence of *B. cereus* especially antibiotic resistant strains indicate poor sanitary conditions during processing which create a health risk for the consumers.

Key words: *Psychrotolerant Bacillus cereus group, food poisoning, ice cream, Bacillus thuringiensis, Antibiotic resistance.*

INTRODUCTION

Bacillus cereus is a facultative anaerobic, gram-positive, catalase-positive, endospore forming, motile organism that consists of six closely related species namely *Bacillus anthracis, Bacillus cereus, Bacillus mycoides, Bacillus pseudomycoides, Bacillus thuringiensis*, and *Bacillus weihenstephanensis* (Ash and Collins, 1992).

B. cereus is widely distributed in nature and is commonly found in a variety of foods of plant and animal origin particularly dairy products. When *B. cereus* grows to high numbers in a food, sufficient enterotoxin may be produced resulting in foodborne illness (Granum, 2002).

B. cereus was not considered as a psychotolerant species until some cold-tolerant isolates had been identified in the 1990s (Van Netten *et al.,* 1990; Dufrenne *et al.,* 1995). Considering the heat resistance of their spores, the vegetative production of emetic and enterotoxic toxins at refrigeration temperatures, the occurrence of psychrotolerant *B. cereus* and their consequent impact on the safety of chilled foods are needed to be investigated.

Likewise, food contamination by *B. cereus* can readily occur because of the ubiquity of the spores, causing them to be almost impossible to be undetected in raw food (Heyndrickx and Scheldeman,

2002). For this reason, contamination may occur after ineffective heat treatment or from packaging problems. Heat treatments such as pasteurization (at least 71.7º C for 15 sec.) efficiently kill vegetative bacteria but not spores.

Usually, two types of *B. cereus* foodborne diseases occur, the diarrhoeal and the emetic types (Stenfors Arnesen *et al.,* 2008). In addition to causing foodborne illness, B. cereus is also capable of causing respiratory tract infections (Gray *et al.,* 1999), mastitis, systemic infection, gangrene, meningitis in immunocompromised children (Gaur *et al.,* 2001) and other clinical problems (Weber, 1988) as a severe form of endophthalmitis can result in loss of functional vision or even blindness (Callegan *et al.,* 2002), urinary tract infections, and fatal liver failure (Kotiranta *et al.,* 2000).

Also, *B. thuringiensis* has been implicated in food-poisoning (Jackson *et al.,* 1995). However, there have been few reports on its prevalence in foods. The potential risks of enterotoxins produced by *B. thuringiensis* have been noted (Jensen *et al.,* 2002; Swiecicka *et al.,* 2006; Ngamwongsatit *et al.,* 2008). It has the same toxicity level as B. cereus isolated from cases of human diarrhoea. Thus, with the present knowledge from B. cereus strains causing diarrhoea, it must be concluded that *B. thuringiensis* has the potential to cause diarrhoea (Hansen and Hendriksen, 2004).

Therefore, this study was conducted for enumeration and identification of psychrotolerant Bacillus cereus group in ice cream, to estimate mainly the relative occurrence of *B. thuringiensis,* as well as, the antibiotic resistance of psychrotolerant *B. cereus* group and *B. thuringiensis* particularly.

MATERIALS and METHODS

Collection of samples:

A total of 150 ice cream samples representing large scale manufacture, small scale manufacture and street vendors (50 samples each), were collected from different supermarkets, shops and street vendors, respectively, in Assiut City.

Samples were collected in ice box and transferred immediately to the laboratory where they were prepared and examined for psychrotolerant *B*acillus cereus group.

Preparation of samples:

Samples were left to melt in a water bath at 44°C for 10 minutes. Each sample was then thoroughly mixed. Eleven ml of the well mixed prepared sample was transferred into a sterile flask containing 99 ml sterile 0.1% peptone water, to obtain a dilution 1/10, then decimal dilutions were prepared as recommended by A.P.H.A. (1992).

Isolation and enumeration of Bacillus cereus group:

According to the U.S. Food and Drug Administration Bacteriological Analytical Manual, (Rhodehamel and Harmon, 2001), isolation and enumeration of Bacillus cereus group were made.

- **Plate count of Bacillus cereus:**

Serial dilutions were prepared from samples. MYP agar plates were inoculated with each dilution of sample. Plates were incubated 24 h at 30°C.

The pure isolated colonies were identified according to the U.S. Food and Drug Administration Bacteriological Analytical Manual (Rhodehamel and Harmon, 2001).

The identification of Bacillus cereus group was by nitrate reduction test, catalase test, anaerobic utilization of glucose, motility test**,** rhizoid growth, hemolytic activity and test for protein toxin crystals. *B. mycoides* characteristically produces rhizoid colonies on agar media. Whereas, *B. thuringiensis* was identified with the test for protein toxin crystals; where nutrient agar slants were inoculated with 24 h culture suspensions. Slants were incubated 24 h at 30°C and then at room temperature 2-3 days. Smears were prepared with sterile distilled water on microscope slides. Slides were air-dried and lightly heat-fixed by passing slide through flame. Slides were stained with methanol then with 0.5% basic fuchsin (Difco). Slides were examined under oil immersion for presence of darkly stained tetragonal (diamond-shaped) toxin crystals. Toxin crystals are usually abundant in a 3- to 4-day-old culture of *B. thuringiensis,* but cannot be detected by the staining technique until lysis of the sporangium has occurred. Therefore, unless free spores can be seen, cultures should be held at room temperature for a few more days and re-examined for toxin crystals. *B. thuringiensis* usually produces protein toxin crystals that can be detected by the staining technique. *B. cereus* and other members of the B. cereus group do not produce protein toxin crystals.

B. cereus was identified as those isolates which are actively motile and strongly haemolytic and do not produce rhizoid colonies or protein toxin crystals.

Antibiotic sensitivity test:

The antibiotic sensitivity was performed using the disc diffusion method according to Bauer *et al.* (1966). A 16 h broth cultures of the collected strains were grown at 37 °C and spread on nutrient agar plate using sterilized glass spreader. Then ampicillin (10 µg), amoxycillin (10 µg), chloramphenical (30 µg), kanamycin (30 µg), cephalexin (30 µg), erythromycin (15 µg), neomycin (30 µg) and streptomycin (10 µg) antibiotic discs were distributed on plate and kept the plates at 4 °C for 4-6 h, so that the antibiotic can diffuse on the agar media.

The plates were then incubated at 37 °C for 16 h and the growth of the bacteria was observed. The presence of a clear zone around the disc was the index of sensitivity to the antibiotic. The test results of antibiotic sensitivity were determined according to the inhibition zone diameter (Barry, 1986). The absence of such a clear zone or the presence of some colonies within the clear zone indicated that the strain was resistant to that antibiotic.

RESULTS

The obtained results were tabulated in Tables 1-5

Table 1: Incidence of psychrotolerant *Bacillus cereus* group in examined ice cream samples.

Sources of examined samples	No. of examined samples	Positive samples	
		No.	%
Large scale produced ice cream	50	10	20
Small scale produced ice cream	50	20	40
Street vendors ice cream	50	18	36
Total	150	48	32

Table 2: Psychrotolerant *Bacillus cereus* group (CFU/gm) in examined ice cream samples.

Sources of examined samples	Min.	Max.	Average
Large scale produced ice cream	$1x10^2$	$1x10^3$	$5\ x10^2$
Small scale produced ice cream	$1x10^3$	$48x10^4$	$24x10^3$
Street vendors ice cream	$1x10^3$	$2x10^4$	$12x10^3$

Table 3: Percentage of ice cream samples contains the infective dose of psychrotolerant *Bacillus cereus* group**.**

Sources of examined samples	$\leq 10^3$ CFU/gm		$> 10^3$ CFU/gm*	
	No.	%	No.	%
Large scale produced ice cream	10	100	0	0
Small scale produced ice cream	10	50	10	50
Street vendors ice cream	10	55.6	8	44.4

***** The infective dose must surpass 10^3 CFU/gm of *B. cereus* according to Granum (2002).

Table 4: Frequency distribution of psychrotolerant *Bacillus cereus* group in examined ice cream samples.

Species	Large scale produced ice cream		Small scale produced ice cream		Street vendors ice cream	
	No.	%	No.	%	No.	%
B. cereus	5/10	50	6/20	30	10/18	55.55
B. mycoides	3/10	30	8/20	40	5/18	27.78
B. thuringiensis	2/10	20	6/20	30	3/18	16.67

Table 5: Antibiotic sensitivity of *B. thuringiensis* isolates

		No. (%)	
Antimicrobial	Conc (μg/disc)	Resistant	Sensitive
Ampicillin	10	8(72.7%)	3(27.3%)
Amoxycillin	10	7(63.6%)	4(36.4%)
Chloramphenical	30	0	11(100%)
Kanamycin	30	0	11(100%)
Cephalexin	30	1(9.1%)	10(90.9%)
Erythromycin	15	2(18.2%)	9(81.8%)
Neomycin	30	11(100%)	0
Streptomycin	10	11(100%)	0

DISCUSSION

This study has shown that B. cereus group can be isolated from the three kinds of ice cream. The possibility of an entry of B. cereus group into ice cream is through the use of contaminated mixtures. Another possibility for the origin of B. cereus group is the use of pasteurized milk in ice cream. Likewise, B. cereus spores can survive the pasteurization process. Therefore, pasteurized milk is a possible vehicle for the entry of B. cereus into ice cream (Novak *et al.,* 2005).

Cold adaptation of contaminated B. cereus group endues the bacteria to grow quickly at low temperature with the consequence of greater potential hazard in chilled food.

The results recorded in Tables 1 and 2 revealed that the psychrotolerant *Bacillus cereus* group were present in 20%, 40% and 36% of examined ice cream samples collected from large scale producers, small scale producers and street vendors, with average values of 5 $x10^2$, $24x10^3$ and $12x10^3$ CFU/gm, respectively.

These results reflect the effect of manufacturing process in ice cream samples on the quality of final products. As the large scale produced ice cream is produced under strict hygienic measures accomplished by efficient packaging, handling, storage and delivery to

consumers. Rather than the small scale produced and street vendors ice cream, where uncontrolled hygienic measures are usual practices reflected by salesmen behaviour during handling and temperature where it is kept, as well as, the process of filling in final containers which are delivered to the consumers considered as a source of contamination (Masud, 1989).

There is currently a consensus with the respect to the dose necessary for a food item to be considered safe for human consumption. This dose should not surpass 10^3 CFU of *B. cereus* in 1 gram or 1 ml of food (Granum, 2002).

According to Granum, 2002, it was found in this investigation as illustrated in Table 3 that 10 samples out of 20 small scale produced ice cream and 8 out of 18 street vendors' ice cream samples were highly contaminated exceeding the infective dose of B. cereus thus may be infective to consumers causing food poisoning if enterotoxins were produced. While, all large scale produced ice cream did not surpass 10^3 CFU/gm and considered safe, this reflects the effect of manufacturing process in ice cream samples on the quality of final products.

Ahmed *et al.* (1983) isolated B. cereus from 48% of ice cream samples. Also, Saleh *et al.* (1993) found B. cereus in 44% of ice cream samples. While, Wong *et al.* (1988) found B. cereus in 52% of ice creams and 35% of soft ice creams. The relatively low incidence of the present study (32%) in total examined ice cream samples (Table 1) compared to other investigations could be attributed to that during the ice cream freezing process; the number of vegetative cells of B. cereus might be reduced to a very low or undetectable level (Messelhäusser *et al.,* 2010).

The use of high heat treatment can result in commercially sterile food products, spoilage can occur quite frequently, because the sporeforming organisms characterized by their high heat resistance and their thermostable protease enzyme lead to spoilage of such contaminated foods (Chopra and Mathur, 1983). Therefore, hygienic measures must be adopted to reduce microbial population and prevention of subsequent contamination of food products during processing and handling.

B. thuringiensis was detected due to the presence of intracellular crystals (Harmon, 1982). It resembled 20% of *B. cereus* group strains

isolated from large scale produced ice cream, while it was 30% and 16.67% in case of B. cereus group strains isolated from small scale produced and street vendors ice cream samples, respectively (Table 4). Despite the pathogenic characteristics of *B. thuringiensis*, the presence of this bacterial species in food and food borne disease is not well described. Earlier studies did not distinguish between *B. thuringiensis* and *B. cereus* probably because of similar methods for detection and enumeration of *B. cereus*-like organisms in food. This observation results in the speculation that *B. thuringiensis* could actually be responsible for many of the foodborne outbreaks previously attributed to *B. cereus*.

Cogan (1980) declared that most psychrotrophic bacteria are readily killed by pasteurization but thermoduric psychrotrophic bacteria still survive. So, it could be stated that the initial raw milk used in manufacturing of ice cream samples was originally highly contaminated with psychrotolerant *Bacillus cereus* group.

Collins (1981) illustrated that clean equipment and cooling will have a great value in control of thermoduric psychrotrophic bacteria in dairy products.

Since *B. cereus* is ubiquitous in the environment, preventing contamination of food with its spores is almost impossible. Thus, measures to inhibit spore germination and prevent the growth of vegetative cells in ready-to-eat foods might be the approach to effectively prevent and control the spread of this pathogen. Thorough cooking is most likely to destroy the vegetative cells and spores. However, temperatures under 100°C might allow spore survival. Cooling should be done rapidly.

Studying the antibiotic sensitivity of *B. thuringiensis* in Table 5, revealed that it was resistant to amoxicillin, ampicillin, streptomycin and neomycin. Whereas, sensitive to erythromycin, chloramphenical, cephalexin and kanamycin. It is nearly similar to that reported by Luna *et al.* (2007). Whereas, Sarker *et al.* (2010) found that *B. thuringiensis* was sensitive to ampicillin and streptomycin.

It is noticeable to mention that the antibiotic sensitivity pattern of psychrotolerant *B. cereus* group isolates was similar to *B. thuringiensis*.

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