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PREVALENCE OF CLOSTRIDIUM SPECIES IN CONCENTRATED AND DRIED MILK

(With 4 Tables)

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(Received at 16/9/2011)

مدى تواجد عترات الكلوستريديم فى اللبن المركز واللبن الجاف

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تم جمع 150 عينة عشوائية من اللبن المبخر، المكثف والمجفف من محلات الألبان والسوبر ماركت من مدينة أسيوط في الفترة من شهر فبراير حتى مايو سنة ٢٠٠٨ بواقع ٥٠ عينة من كل منتج لمعرفة مدي تلوثها بالبكتريا اللاهوائية وقد أسفرت النتائج على أن نسبة تواجد Clostridium species هي ٤٠ ، ٤٠ و ٩٨% ومتوسط العدد الكلي ١.٥ x ١٠^{٢} ، ٤ x ١٠^{٣} و ٨.٥ x ١٠^{٢} في العينات المفحوصة علي التوالي. كما تم عزل عدة أنواع مثل Cl. ramosum, Cl.perfringens, Cl. difficile, Cl. innoculum, Cl. septicum, Cl. novyi type A, Cl. cadaveris, Cl. subterminale بنسب مختلفة ، بينما لم يستدل علي وجود Cl.botulinum في جميع العينات التي تم إختبارها على الترتيب. علاوة على ذلك كان متوسط العدد الكلي لـ Cl. perfringens هو أقل من ١٠، ٠ و ١.٥x ١٠^{٢} لكل جرام باستخدام طريقة MPN. بالأضافة إلى هذا فقد تم فحص ١٠ عترات من ميكروب Cl. perfringens بواقع ٨ عترات من اللبن المجفف و ٢ عترة من اللبن المبخر لتحديد السموم الداخلية المفرزة بواسطة الإليزا وكانت جميعها سلبية لإفراز سموم ألفا. ونوقشت الأهمية الصحية لهذه الميكروبات وخطورة تواجدها خاصة فى الألبان المركزة والجافة التى يعتمد عليها الكثير من الأطفال الرضع ومراعاة تطبيق نظام الهاسب ومراقبة الجودة فى مصانع الأنتاج.

SUMMARY

A total of 150 random samples of evaporated; condensed and dried milk (50 samples each) were collected from dairy shops and supermarkets in Assiut city for the presence of anaerobes. By using plate method the percentages were 40, 40 and 98 % with an average count of 1.5×10^{2}; 4

× 10 ^{3} and 8.5 × 10 ^{2} /g; respectively. *Cl.species* as *Cl.ramosum, Cl.perfringens, Cl.difficile Cl.innoculum, Cl.septicum, Cl.novyi type A, Cl.cadaveris and Cl.subterminale* were identified in varies percentages from the evaluated samples. In addition, *Cl. perfringens* was recorded in a percentage of 16 % with an average count of 1.5×10^{2}/g of dried milk using MPN technique. Fortunately, *Cl.botulinum* failed to be detected in all examined samples. Moreover, 8 and 2 *Cl.perfringens* isolated, respectively from dried and evaporated milk were negative for α-Toxin produced by *Cl.perfringens* by using Enzyme linked immunosorbent assay (ELISA). Public health as well as economical significance of detected microorganisms in the concentrated and dried milk were discussed.

Key words: *Prevalence, Clostridium species, concentrated milk, dried milk, ELISA.*

INTRODUCTION

The consumer awareness has played a predominant role in emphasizing the need for microbiologically safe foods for human consumption. Serious health hazards due to the presence of pathogenic microbes in milk products provide a highly favourable media for multiplication of different types of food poisoning microorganisms from different sources**.** The presence of anaerobes in dairy products is no assurance that they well develop but it would appear that if large numbers were involved the chance for their growth would be better than if only small numbers were included. Their presence is more difficult to control, especially in dairy industry due to several reasons. First of all it seems to be impossible to completely avoid the presence of anaerobic spores in all milk samples. Secondary, the spores are very hydrophobic and will attach to the surface of pipelines of the dairy plant, where they might multiply and repopulate. A third problem is that pasteurization heating is insufficient to kill the spores, while competition from other vegetative bacteria is eliminated (Andersson *et al.,* 1995).

Cl.perfringens has the ability to grow at high temperatures very rapidly causing problems in foods that are not cooled quickly. Its optimum growth temperature is 43-45^{o}C which has one of the fastest known growths rates for any bacterium. It has the potential to grow between 15 and 50^{o}C and its vegetative cells are usually destroyed at 60^{o}C and above. Spores present in food from the environment can very

considerably in their heat resistance, surviving at temperature from 95 - 100^{o}C for periods of up to one hour (McClane, 1997). Enterotoxigenic *Cl.perfringens* strains are also associated with non-foodborne digestive diseases such as antibiotic associated diarrhoea, chronic non-foodborne diarrhoea, and some cases of sudden infant death syndrome. Immunological immaturity of some infants could lead a non-selective absorption of molecules including *Cl.perfringens* enterotoxin from the intestine and to a rapid transport the circulation responsible for the systemic effects of *Cl.perfringens* enterotoxin (Petit *et al.,* 1999).

Currently, there is a need for a rapid, simple and sensitive serological assay for large-scale detection of *Cl.perfringens* enterotoxin. Enzyme-linked immunosorbent assay (ELISA) method potentially satisfy these needs, and ELISA have been developed for the specific detection of several bacterial toxins, including cholera enterotoxin, *E.coli* heat -labile enterotoxin, *Cl.botulinum* type G-toxin and *Cl.difficile* toxin A (McClane and Strouse, 1984).

Therefore, this work was planned to determine the rate of contamination of condensed; evaporated and dried milk with *Clostridium species* to explore their public health and economic importance. This include enumeration; isolation and identification of *Clostridium species* as well as the detection of *Cl.perfringens* type A enterotoxines by using Enzyme linked immunosorbent assay (ELISA).

MATERIALS and METHODS

Part I: Detection of anaerobic bacteria:

A total of 150 random samples of evaporated, condensed and dried milk (50 samples each) were purchased from dairy shops and supermarkets in Assiut city, Egypt. The samples were still valid for consumption. All samples were delivered promptly to the laboratory with a minimum of delay for bacteriological examination.

1- Dried milk samples were prepared as described by L.M.B.G. (1991).
2- Concentrated milk cans were prepared according to A.P.H.A. (1992).

Bacteriological examination:-

I- Enumeration of *Clostridium species* by plate method using Reinforced Clostridial agar (RCM) as described by I.C.M.S.F. (1978). Suspected colonies of characteristic shape and yellowish colour were calculated as presumptive count.

II- Isolation of *Clostridium species* according to Wen and McClane (2004).

III- Enumeration of *Cl. perfringens* by MPN technique using lactose sulphite broth (LSB) (Beerens et al., 1980). The counts of *Cl.perfringens* were recorded by using MPN Tables.

VI- Identification of isolates by**:-**

1- Morphological characters include: - Microscopical appearance using Gram's stain, motility test and cultural characters, where size and shape of the colonies, type of haemolysis on blood agar and change in meat particle were recorded (Holt et al., 1994).

2- Biochemical reactions include:- Sugar fermentation, indol production, changes in litmus milk, gelatin liquefaction, haemolysis on neomycin blood agar, nitrate reduction and changes in iron milk (Krieg and Holt, 1984)

3- Reaction on egg yolk media as described by Walker (1990).

Part II: Detection the enterotoxigencity of *Cl.perfringens* type A by using ELISA.

This part has been done in the Genetic Engineering and Molecular Biology Research Centre in Assiut University Egypt, using Bio-x Alpha toxin ELISA kit (Biok 084) (Bio-X Diagnostics-Belgique, 2008). Selected strains of the isolated *Cl.perfringens* from the examined samples were tested for their ability to produce enterotoxins.

RESULTS

The obtained results were recorded in Tables 1-4.

Table 1: Statistical analytical results of Clostridium species count / g of examined samples using surface plating technique.

Examined samples	Positive samples		Count / g		
	No./ 50	%	Min.	Max.	Average of +ve samples
Evaporated milk	20	40	*<10^2	2.0 ×10^2	1.5 ×10^2
Condensed milk	20	40	*<10^2	1.1 ×10^4	4.0 × 10^3
Dried milk	49	98	*<10^2	8.0 ×10^3	8.5×10^2

* Colonies could not be detected on the plates.

Table 2: Incidence of different Clostridium species in the examined samples using surface plating technique.

Clostridium species	Evaporated milk		Condensed milk		Dried milk	
	Positive samples		Positive samples		Positive samples	
	No. / 50	%	No. / 50	%	No. / 50	%
Cl. ramosum	5	10	3	6	15	30
Cl. perfringens	-	-	-	-	7	14
Cl. difficile	13	26	2	4	11	22
Cl. innoculum	2	4	14	28	4	8
Cl. septicum	-	-	-	-	10	20
Cl. novyi type A	-	-	-	-	1	2
Cl. cadaveris	-	-	1	2	-	-
Cl. sporogenes	-	-	-	-	-	-
Cl. hastiforme	-	-	-	-	-	-
Cl. butyricum	-	-	-	-	-	-
Cl. subterminale	-	-	-	-	1	2

Table 3: Statistical analytical results of Clostridium perfringens count in the positive samples using MPN technique/g.

Examined samples	Positive samples		Count / g		
	No./50	%	Min.	Max.	Average of +ve samples
Evaporated milk	2	4	<10	<10	<10
Condensed milk	-	-	-	-	-
Dried milk	8	16	< 10	1.1×10^3	1.5×10^2

Table 4: Detection of Alpha Toxin by Elisa in the examined samples.

Examined samples	No. of examined samples	Positive α-Toxin		Negative α-Toxin	
		No.	%	No.	%
Dried milk	8	0	0	8	100
Evaporated milk	2	0	0	2	100
Total	10			10	

DISCUSSION

I- Enumeration of Clostridium species by using plate method technique.

The incidence of *Clostridium* organisms in examined samples of evaporated milk were 40% (Table 1) at level varied from $< 10^2$ to 2×10^2/g with an average count of 1.5×10^2/g. This finding was higher than that recorded by Amer and El-Mossalami (2006); Edema and Akingbade (2007).

In case of condensed milk, the incidence of *Clostridium* organism was 40%. The minimum count was $< 10^2$/g, a maximum was 1.1×10^4/g and the average count were 4×10^3/g. These records were higher than those obtained by Mohamed (1984); Korashy and Sabreen (2001); Amer and El-Mossalami (2006). In general, sweetened condensed milk is not a sterile product, and the various methods of heat treatment used are not adequate to kill sporeforming bacteria and further processing and handling usually contribute a variety of microorganisms besides the sugar levels employed permit some types to grow if other conditions are favourable. Enough oxygen may be present in the headspace of an incompletely filled or poorly sealed container, to permit the growth of organisms able to tolerate the high osmotic pressure of the product.

Regarding dried milk, *Clostridium species* detected in 49(98%) samples, in count ranged from $< 10^2$ to 8×10^3/g with an average value of 8.5×10^2/g.

Recorded results were higher than that reported by El-Bassiony and Aboul-Kheir (1983); Hafez and Ahmed (1988); El-Prince and Korashy (2003) however, lower than the recorded results by Lück *et al.* (1980). The microbial counts of dried milk depend upon the temperature, time of preheating of milk, evaporation process if used, contamination growth in storage tanks and pipes and method of drying and packaging process.

II- Incidences of different *Clostridium species*:

It is evident from the findings in Table 2, that *Clostridium species* were present in evaporated milk samples. The percentages of *Cl.ramosum, Cl.difficile* and *Cl.innoculum* were 10, 26 and 4%, respectively, however, *Cl.perfringens* failed to be detected.

The presence of these microorganisms implies that, primary contamination of the product may have occurred at manufacturing sites which is in agreement with Mahari and Gashe (1990). They stated that contamination at factory could occur through air in the holding tank and pipes or the pasteurization room. Moreover, the clostridia spores were able to survive the temperature used for pasteurization of milk to some extent and later germinate and multiply in milk above 60^{o}C (Griffiths, 1992). Furthermore, contamination of milk products could be incriminated to various roles performed by workers during milk processing (Gill *et al.,* 1994).

In case of condensed milk, the *Cl.ramosum, Cl.difficile, Cl.innoculum,* and *Cl.cadaveris* were recovered from 3(6%), 2(4%), 14(28%) and 1(2%) samples, respectively. These findings are in agreement with those postulated by Amer and El-Mossalami (2006), while, Korashy and Sabreen (2001) could isolate *Cl.perfringens*.

Using high quality raw milk and fine clean sugar in condensed milk manufacture, good sanitation and hygiene during production, handling and storage are important to prevent the condensed milk from spoilage and to protect consumers from infection. In addition, high standard of plant hygiene is needed to avoid post-processing contamination. However, the heat treatments used in its production are insufficient to sterilize the product, so strict hygienic measures are still required. Also, the viscous nature of this product needs to be taken into account for the cleaning procedures. Finally, employment of experienced staff is necessary at all times.

The results postulated in Table 2 indicated that incidences of *Cl. ramosum, Cl. perfringens, Cl. difficile, Cl. innoculum, Cl. septicum , Cl. novyi type A* and *Cl. subterminale* in the evaluated dried milk samples were 15(30%), 7(14%), 11(22%), 4(8%), 10(20%), 1(2%) and1(2%), respectively. The results of *Cl. perfringens* were in harmony with that obtained by Mahmoud (1987); Hafez and Ahmed (1988). Higher findings were recorded by Abd El-Hakiem (1992); Wahba (1997). However, lower records estimated by El-Leboudy (1985); Ahmed and Abd El-Gaber (1994); Saad (1995). With regard to *Cl. cadaveris* higher finding was detected by Hafez and Ahmed (1988).

The presence of *Cl. perfringens* in milk powder may be attributed to contaminated raw milk used. Also, the incidence of such organisms in dried milk reflect the hygienic measures applied during production, handling, manufacturing and distribution and these products are considered to be of poor quality or even unfit for human consumption. Furthermore, the presence of such organisms in large numbers may constitute a public health hazard.

III- Incidence and count of *Cl. perfringens* using MPN technique.

It is apparent from the recorded data in Table 3 that; two samples (4%) of evaporated milk were contaminated with *Cl. perfringens* in count less than 10/g. This was nearly parallel to that reported by Amer and El-Mossalami (2006). However, Edema and Akingbade (2007) indicated higher result. In contrast, *Cl.perfringens* failed to be detected in condensed milk which was in agreement with Amer and El-Mossalami (2006). Higher incidence was estimated by Korashy and Sabreen (2001).

Furthermore, in dried milk the concerning organism was detected in 8 (16%) of the examined samples in counts ranged from < 10 to 1.1×10^3 with an average of 1.5×10^2 /g. With regard to the count obtained, it was higher than that registered by Lück *et al.* (1980); Hafez and Ahmed (1988); Abd El-Hakiem (1992); Ahmed and Abd El-Gaber (1994); Saad (1995).

IV- Detection of *Clostridium perfringens* type A enterotoxins by using enzyme linked immunosorbent assay (ELISA).

It is often difficult to distinguish *Cl.perfringens* food poisoning without laboratory evidence, bacteriological examination are usually employed to diagnose this food poisoning. Since up to 95% of normal adults have *Cl.perfringens* in their faecal flora. Its isolation without

epidemiological or other support is not sufficient for diagnosis. Currently, it is desirable to develop more rapid and convenient method for diagnosis of *Cl.perfringens* food poisoning. Several methods of its enterotoxins detection have been reported, but none has yet been extensively used for the investigation of food-borne disease outbreaks, this is at least partially due to the inconvenience of these assays for large scale application. Additionally, some assays are unsatisfactory for investigation food-borne disease, owing to interference by faecal material (McClane and McDonel, 1981). Elisa procedures are rapid, specify and sensitive serological assays which have been used for detection of several bacterial toxin (Ketyi and Pacsa, 1980; Lewis *et al.,* 1981; Lyerly *et al.,* 1983).

In this study, Bio-X Alpha Toxin Elisa Kit (BioK084) was used for detection of *Cl.perfringens* Alpha Toxin in culture supernatants. It is produced in varying amount by all biotypes (A, B, C, D, and E) of *Cl.perfringens* and considered a primary virulence factor involved in *Clostridial myonecrosi* (Table 4).

Alpha Toxin is the only lethal protein produced during vegetative growth of type A strains, the most ubiquitous *Cl.perfringens* biotype that is commonly found in soil and normal intestinal flora of humans and animals. Due to its rule in gas gangrene, food poisoning and animal enterotoxima *Cl.perfringens* type A strain, particularly the Alpha Toxin have been the subject of intense investigations over the past 60 years (McDonel, 1986).

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