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INCIDENCE OF CAMPYLOBACTER SPECIES IN MILK AND SOME MILK PRODUCTS

(With 6 Tables and One Figure)

By

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(Received at 30/5/2007)

مدى تواجد ميكروبات الكامبيلوباكتر فى اللبن وبعض منتجات الألبان

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تعتبر الألبان ومنتجاتها عرضة للتلوث أثناء صناعتها وتداولها بعديد من الميكروبات الممرضة التى تمثل خطراً على صحة المستهلك ويأتى فى مقدمة تلك الميكروبات ميكروب الكامبيلوباكتر الذى يؤدى إلى حدوث مغص قولونى مصحوب بإسهال مدمم. لذلك قد تم عمل هذه الدراسة لمعرفة مدى تلوث الألبان ومنتجاتها بهذا الميكروب وقد اشتملت الدراسة على ٣٥٠ عينة عشوائية تمثلت فى ١٥٠ عينة من اللبن الخام، ٥٠ عينة من كل من الجبن الدمياطى، الجبن القريش، والأيس كريم، الزبد الفلاحى. وقد تبين بالفحص أن ١٠ (٦,٧%) ، ٧ (١٤%) و ٥ (١٠%) من عينات اللبن والجبن القريش والأيس كريم كانت ملوثة بميكروبات الكامبيلوباكتر وذلك باستخدام مستنبت Brucella agar. أما باستخدام مستنبت Campylobacter agar وجد أن ٩ (٦%) ، ٣ (٦%) ، ٣ (٦%) ، ١ (٢%) و٣ (٦%) من عينات اللبن ، الجبن القريش ، الأيس كريم ، الجبن الدمياطى والزبد على التوالى كانت ملوثة بميكروبات الكامبيلوباكتر. كذلك تم عزل وتصنيف ميكروبات C. jejuni ، C. coli ، C. laridis ، C. fetus ، C. hyointestinalis C. fecalis بنسب مختلفة من العينات المفحوصة. وقد أعزى هذا التلوث إلى عدم إتباع القواعد الصحية السليمة أثناء إنتاج اللبن وتوزيعه وكذلك أثناء تصنيعه حتى يصل إلى المستهلك بينما وجد أن تخزين الجبن الدمياطى وانخفاض التركيز الأيونى الهيدروجينى به أدى إلى الحد من نمو الميكروب بها. وباستخدام التحليل الالكتروفوريسى تبين أن خمسة من عشرة معزولات من ميكروب C. jejuni كانت تحمل بلازميدات ذات أحجام جزيئية عالية. وقد أسفرت النتائج عن وجود بعض المقاومة من الميكروب الذى يحمل بلازميد لكل من Cephalothin, Kanamycin, Flemkuin and Oxytetracyclin كما أسفرت النتائج عن حساسية الميكروب بنسبة ١٠٠% لكل من Enrofloxacin, Norfloxacin Nalidixic acid, Gentamycin. هذا

وقد تم وضع الشروط والضوابط الصحية وكذلك الخطوات التى يجب إتباعها أثناء إنتاج وتصنيع وتداول الألبان ومنتجاتها لتجنب التلوث بهذا الميكروب.

SUMMARY

Three hundred and fifty random samples of raw milk (150) and some milk products including kareish and Damietta cheese, ice-cream and cooking butter (50 samples each) were collected from Assiut city Markets, dairy shops, and dairy farms. The samples were examined for isolation and identification of Campylobacter spp. The obtained results revealed that 10 (6.7%), 7 (14%), 5 (10%) of the examined raw milk, kareish cheese and ice cream samples were contaminated by Campylobacter spp. using Brucella agar medium. However, the incidence of Campylobacter spp. Using Campylobacter agar was 9 (6%) in raw milk samples, 3 (6%) in kareish cheese, 6 (6%) in ice cream, 1 (2%) in Damietta cheese and 3 (6%) in cooking butter. The isolated Campylobacter spp. could be identified as Campylobacter jejuni, C. coli, C. laridis, C. fetus, C. hyointestinalis and C. fecalis. Plasmid profile and antibiogram of the isolated Campylobacter jejuni recovered from the examined raw milk and dairy products revealed that 5 out of 10 isolates (50%) of C. jejuni carry (1-2) plasmids of high molecular weight with resistance to Cephalothin, Oxytetracycline, Flemkuin and Kanamycin and sensitivity to Norfloxacin, Enrofloaxacin, Gentamycin and Nalidixic acid. The bublic heath significance and suggestive measures to improve the keeping quality as well as sanitary conditions of milk and milk products were given.

Key words: *Campylobacter spp, milk, milk products, plasmid, antibiotic sensitivity.*

INTRODUCTION

In the last few years Campylobacters have emerged as the most frequent cause of acute bacterial gastroenteritis in man (Casini *et al.*, 1997) as the number of human cases of Campylobacteriosis has increased dramatically in recent years in many countries (Nielsen *et al.*, 2000).

The term Campylobacter is a Greek name composed of campy, means curved and bacter, means rod i.e curved rods which describes the appearance of the organisms (Nachamkin *et al.*, 1992).

The genus Campylobacter comprises many species (14 species, subspecies, biovars, with 17 official names) (Abdel-Samei, 2000), which are newly established as bacterial agents of clinical importance in humans. The most frequently identified human pathogenic species of Campylobacter are C. jejuni and C. coli which are closely related and their infections appear to share many clinical and epidemiologic characteristic in addition to C. laridis. They account for more than 99% of the human isolates of infection (C. jejuni 90%). Recent work suggests that C. upsaliensis is also enteropathogenic, and occasionally other species such as C. hyointestinalis was isolated from patients with diarrhea. (Fennell *et al.*, 1984; Edmonds *et al.*, 1987 and Skirrow, 1990).

Campylobacter may found as a normal intestinal flora of both wild and domesticated animals specially those used for food production (Penner, 1988). However,the main source of Campylobacter infection is probably raw milk and milk products which are the most commonly implicated vehicles in food - borne outbreaks of Campylobacter enteritis. (Richter *et al.*, 1992 and Bean *et al.*, 1996). Contamination of milk can occur by direct excretion from an asymptomatic cow with mastitis (Hutchinson *et al.*, 1985) or through fecal contamination during milking from cattle infected or colonized with the organism (Waterman *et al.*, 1984 and Humphrey and Beckett 1987). Also post pasteurization contamination of milk and dairy products have been found responsible for Campylobacter outbreaks (White, 1986).

The mechanism of Campylobacter pathogenesis has not been elucidated fully, as several virulence related factors such as adherence, invasiveness and production of heat labile cholera-like enterotoxin or cytotoxin have been associated with the organism (Walker *et al.*, 1986 and Kalman *et al.*, 2000). C. jejuni has become the most commonly reported cause of food-borne enteritis in people world wide (Skirrow, 1990 and Butzler and Oosterom, 1991). It can produce different types of toxins as enterotoxin, cytolethal distending toxin and cytolethal rounding toxin.

The infective dose of C. jejuni ranges from 500 to 10.000 cells, (Doyle, 1991; Reed, 1994 and Phillips, 1995). Campylobacter enteritis has been associated with some complications such as arthritis, recurrent colitis, Heamolytic Uremic Syndrome (HUS), Reiter syndrome, a reactive arthropathy which develops in about 1% of patients 1-2 weeks after onset of illness, Miller-fisher syndrome, Chinese paralytic syndrome, Guillian-Barre syndrome (GBS) particularly in immunocompromized individuals, which is, a disorder resulting in acute

neuromuscular paralysis, as a serious sequelae of Campylobacter infection, up to 40% of patients with GBS have evidence of recent Campylobacter infection (Smith, 1995 and Allos, 1997).

Although Campylobacter doesn't commonly cause death, it has been estimated that approximately 200 persons with Campylobacter infections may die each year in the United States (Skirrow 1990 and Tauxe 1992).The vast majority of outbreaks of Campylobacteriosis have been associated with consumption of unpasteurized or inadequately pasteurized cow's milk in New Zealand, Scotland, Switzerland and England (Hutchinson *et al.*, 1985; Hudson *et al.*, 1990) as well as dairy products (Barrett, 1986). In Egypt, acute diarrhea of presumed infectious origin is responsible for more than 50% of deaths for those under two years of age (Ewyda, 1990).

Therefore, Campylobacter spp. continue to be highly important human pathogens and there is an increase interest of Campylobacters particularly C. jejuni as a health risk affecting both human and animal and because of the involvement of milk and milk products in human Campylobacter enteritis, the present work was planned to study the following items:

- Occurrence of Campylobacter spp. in milk and some milk products.
- Identification of the isolated Campylobacter spp.
- Plasmid profile and antibiotic sensitivity of the isolated strains of C. jejuni.

MATERIALS and METHODS

1. Isolation of Campylobacter spp. from milk and some milk products:

1. Collection of samples:

A total of 350 random samples of raw milk (150), (Damietta and kareish cheese), ice-cream and cooking butter (50 samples each) were purchased from different localities in Assiut city. The samples were collected in clean, dry and sterile containers while, ice cream-samples were taken in an ice-box. Collected samples were transferred to the laboratory as soon as possible to be examined. Each milk sample was mixed by inversion several times and tested for heat treatment using Storch's test (Lampert, 1975).

2. Preparation of samples: The samples were prepared according to the technique recommended by (A.P.H.A., 1992).

3. Experimental procedures: The technique adopted by Boer *et al.* (1984) was used.

3.1.1- Enrichment procedure:

1 ml of each prepared sample was inoculated into Campylobacter enrichment broth and brucella broth. Each broth containing 5% lysed horse blood, Skirrow Campylobacter selective supplement and Skirrow Campylobacter growth supplement. The inoculated tubes were incubated at 42°C for 24 h in an atmosphere of 5% oxygen, 10% carbon dioxide and 85% nitrogen using an anaerobic jar and Campylobacter gas generating kits (Oxoid, 1990).

3.1. 2- Selective plating:

Incubated broth cultures were then streaked onto plates of both Brucella and Campylobacter blood agar base supplemented with Skirrow Campylobacter selective supplement, 5% lysed horse blood, and Skirrow Campylobacter growth supplement. Streaked plates were incubated at 42°C for 48 h under appropriate microaerophilic conditions in anaerobic jar with activated gas generating kit by using the Gas pack system BBL.

All pure cultured colonies were subjected to identification scheme according to Skirrow (1990), Rosef and Yundestad (1982) and Smith *et al.* (1997).

II. Plasmid DNA Extraction:

It has been carried out in the Molecular Biology and Genetic Engineering Research Center in Assiut University. Detection of plasmid DNA was done by Agarose Gel Electrophoresis; (Kaufman *et al.*, 1995).

III. Antimicrobial sensitivity testing of Campylobacter jejuni: Using disc diffusion method (Baron *et al.*, 1994)

RESULTS

Table 1: Incidence of campylobacter spp. in the examined samples of milk and milk products.

Examined samples	*No. of samples*	*Positive samples*			
		Brucella agar		*Campylobacter agar*	
		No.	*%*	*No.*	*%*
Milk	150	10	6.7	9	6
Damietta cheese	50	-	-	1	2
Kareish cheese	50	7	14	3	6
Ice – cream	50	5	10	3	6
Butter	50	-	-	3	6
Total	350	22	6.3	19	5.4 %

Table 2: Incidence of C. jejuni in the examined samples of milk and milk products.

Examined samples	*No. of samples*	*Positive samples*			
		Brucella agar		*Campylobacter agar*	
		No.	*%*	*No.*	*%*
Milk	150	2	1.3	5	3.3
Damietta cheese	50	-	-	1	2
Kareish cheese	50	1	2	1	2
Ice – cream	50	1	2	3	6
Butter	50	-	-	-	-
Total	350	4	1.1 %	10	2.9 %

Table 3: Incidence of campylobacter spp. in the examined milk and milk products samples using Brucella agar medium.

Examined samples	No. of samples	Total isolates	No. of identified											
			c. jejuni		C. coli		C. fetus		C. laridis		C. hyointestinalis		C. fecalis	
			No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Milk	150	10	2	1.3	2	1.3	2	1.3	3	2	1	0.07	-	-
Damietta cheese	50	-	-	-	-	-	-	-	-	-	-	-	-	-
Kareish cheese	50	7	1	2	3	6	-	-	-	-	1	2	2	4
Ice – cream	50	5	1	2	3	6	-	-	-	-	1	2	-	-
Butter	50	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 4: Incidence of campylobacter spp. in the examined milk and milk products samples using Campylobacter agar medium.

Examined samples	No. of samples	Total isolates	No. of identified isolates											
			c. jejuni		C. coli		C. fetus		C. laridis		C. hyointestinalis		C. fecalis	
			No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Milk	150	9	5	3.3	4	2.7	-	-	-	-	-	-	-	-
Damietta cheese	50	1	1	2	-	-	-	-	-	-	-	-	-	-
Kareish cheese	50	3	1	2	2	4	-	-	-	-	-	-	-	-
Ice – cream	50	3	3	6	-	-	-	-	-	-	-	-	-	-
Butter	50	3	-	-	2	4	-	-	1	2	-	-	-	-

Table 5: Correlation between C. jejuni isolates and plasmids

Sources of isolates	No. of isolates	Plasmid bearing isolates		Non plasmid bearing isolates	
		No. / 10	%	No. / 10	%
Milk	5	2	20	3	30
Damietta cheese	1	1	10	-	-
Kareish cheese	1	1	10	-	-
Ice – cream	3	1	10	2	20
Total	10	5	50%	5	50%

Table 6: Antibiotic sensitivity of Compylobacter jejuni isolated from the examined raw milk and milk products samples.

Types of antibiotics	Degree of sensitivity of campylobacter jejuni isolates	% of sensitivity
Amoxicillin 30 mg	Moderately sensitive (++)	80%
Cephalothin 30 mg	Resistant	0
Enrofloxacin 5 mg	Moderately sensitive (++)	100%
Flemkuin 30 mg	Weakly sensitive (+)	40%
Gentamycin 10 mg	Moderately sensitive (++)	100%
Kanamycin 30 mg	Weakly sensitive (+)	80%
Naldixic acid 30 mg	Sensitive (+++)	100%
Norfloxacin 10 mg	Sensitive (+++)	100%
Oxytetracycline 30 mg	Weakly sensitive (+)	60%

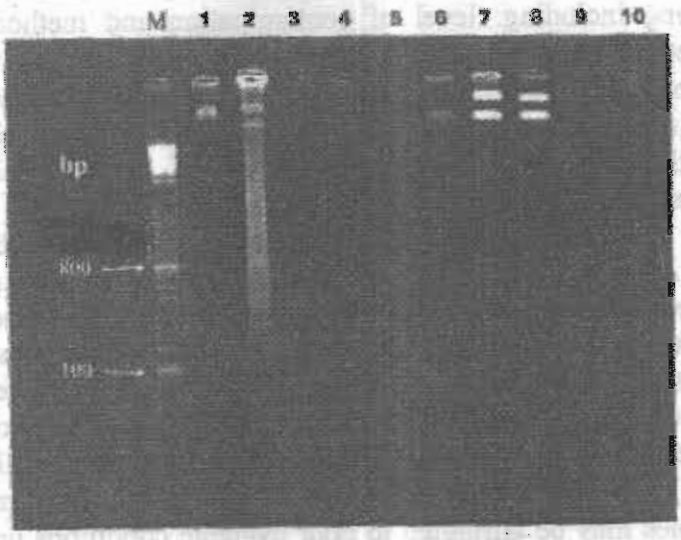

Fig. 1: A garose gel (0.7%) stained with ethidium bromide 0.05% showing:

M : Hundred base pair ladder marker (Sigma)
Lanes 2.7 and 8 : Two copy plasmids of high molecular weight.
Lanes 1 and 6 : single copy plasmid of high molecular weight.
Lanes 3, 4, 5, 9 and 10 showing negative plasmid bearing

DISCUSSION

1- Incidence of Campylobacter spp. in milk and some milk products:

1) Raw milk:

Results recorded in Table 1 showed that Campylobacter spp. were isolated from 10 (6.7%) of 150 examined raw milk samples on Brucella agar and from 9 samples (6%) on Campylobacter blood agar.

These results are nearly in agreement with those obtained by Wegmuiler *et al.* (1993) and Abdel-Hady (1996). Lower percentages were stated by Inokova and Ivanova (1996); Hudson *et al.* (1999) and DuzGun *et al.* (2000). Whereas, higher percentages were recorded by El-Nokrashy *et al.* (1997); El-Prince *et al.* (1998) and Roshdy (2000). However, several investigators failed to detect Campylobacter spp. in the examined milk samples such as Mouffok and Lebres (1992) and Federighi *et al.* (1999).

The variation in these results may be attributed to different factors, including: level of contamination and methods of isolation, variety of enrichment broth systems, high sensitivity of the organism to normal atmospheric concentration of oxygen and to adverse conditions resulting from acid development in raw milk that represent stress factor on the organism resulting in failure of cultural trials even from contaminated samples. (Ray and Johnson, 1984).

According to the results presented in Tables 2 & 3 it is evident that different Campylobacter organisms could be isolated in variant percentages from the examined raw milk samples, these organisms 10 isolates recovered on Brucella agar were identified as C. jejuni (2 isolates), C. coli (2 isolates), C. laridis (3 isolates), C. fetus (2 isolates) and C. hyointestinalis (1 isolate). While, on Campylobacter agar, 9 isolates were identified as C. jejuni (5 isolates) and C. coli (4 isolates).

The presence of Campylobacter spp. in the examined raw milk samples may be attributed to poor hygienic conditions under which raw milk is produced and handled, contamination of milk during or after milking is probably of fecal origin, however improper washing and treatment of the udder with suitable disinfectant or contact of the milking pails with the floor may result in a high level of contamination. Furthermore, naturally occurring Campylobacter mastitis and contaminated water supply may act as a source of milk contamination (Mentzing, 1981).

2) Damietta cheese:

As recorded in Tables 1 & 3 it is apparent that out of 50 examined samples of Damietta cheese using Campylobacter agar, one sample was positive for Campylobacter spp. represented by C. jejuni (2%). This result was in agreement with that obtained by El-Nokrashy *et al.* (1998). Several investigators failed to isolate Campylobacter spp. from the examined cheese samples (Ehlers *et al.*, 1982, Bachmann, 1994 and Federighi *et al.*, 1999).

Lower incidence or absence of the organism in a such product may be due to its fragile nature and sensitivity to adverse conditions of acid development as Campylobacters are inactivated at pH 4.5 (Doyle and Roman, 1981). Water activity and presence of salt (5-15%) which represent stress factors on the organism oftenly result in failure to culture it even from contaminated products or to be recorded as low incidence (Ray and Johnson, 1984). Moreover, Frazier and Westhoff (1988) reported that salt causes high osmotic pressure and hence plasmolysis of cells, dehydrates cheese by drawing out and tying up

moisture as it dehydrates microbial cell and it ionizes to yield the chlorine ion, which is harmful to the organism and it interferes with the action of proteolytic enzymes.

3) Kareish cheese:

The results in Table 1 revealed that Campylobacter spp. were isolated from 7 (14%) of 50 examined kareish cheese samples. According to the results represented in Table 3, it is evident that different Campylobacter organisms could be isolated in variant percentages from the examined kareish cheese samples. These organisms (7 isolates) were identified as C.jejuni (1 isolate) (2%), C. coli (3 isolates) (6%), C. fecalis (2 isolates) (4%) and C. hyointestinalis (1 isolate) (2%) on Brucella agar, while, on Campylobacter blood agar, Campylobacter spp. was isolated from 3 (6%) of the examined kareish cheese samples, these organisms were identified as 1 (2%) C. jejuni and 2 (4%) C. coli as recorded in Table 4. Campylobacter spp. failed to recover from Kareish cheese samples examined by Abdel-Hady (1993) and Federighi *et al.* (1999).

As Campylobacter can remain viable in fresh cheese for only a short period of time (Butzler and Oosterom, 1991). The fact that these cheeses may be consumed immediately after production and may pose a public health risk. The relatively high results of this study could be attributed to the neglected sanitary control adopted during manufacturing, handling and distribution of kareish cheese.

4) Ice-cream:

The data presented in Tables 1 & 3 showed that out of 50 examined samples of ice-cream, 5 samples (10%) contained Campylobacter spp, on Brucella agar, these spp. could be identified as 1 (2%) C. jejuni, 3 (6%) C. coli and 1 (2%) C. hyointestinalis. Whereas, on Campylobacter agar Campylobacter spp. was isolated from 3 (6%) of the examined samples represented by C. jejuni. (Table 4) Several investigators failed to isolate Campylobacter spp. from ice cream (Ehlers et al., 1982; Ray and Johnson, (1984) and Abdel-Hady, 1993). The lower incidence of the organism in such products could be attributed to its sensitivity to conditions of freezing which stressed Campylobacter organisms and resulted in failure to recover the organism from contaminated frozen food.

5) Butter:

Regarding the results in Tables 1 & 3 out of 50 examined samples of cooking butter 3 (6%) were contaminated with Campylobacter spp. These spp. were identified as 2 (4%) C. coli and 1

(2%) C. laridis. The presence of Campylobacter spp. in examined cooking butter samples may be due to poor hygienic conditions under which butter is produced and handled. Such contamination is probably of fecal origin.

IV- Plasmid profile and antibiotic susceptibility of Campylobacter jejuni:

Bacterial plasmids are extrachromosomal DNA known to be code for toxin production, adhesiveness, antibiotic resistance and serum resistance (Baroun and Ou, 1991 and Lax *et al.*, 1995).

Plasmid analysis was performed in the present study on C. jejuni isolated from raw milk and milk products (Damietta cheese, kareish cheese and ice-cream) as well as their sensitivity to some selected antibiotics. The plasmid pattern in Table 5 of the examined strains belonging to C. jejuni showed that 5 (50%) out of the 10 strains bear plasmids of high molecular weight (over 2.6 Kpb) 3 of the 5 isolates carry 2 copies of plasmid as showed in Fig.1, higher percentages were detected by Ansary and Veloo (1991) (62.2%) and Jay (1996) (64.7%). Lower percentages were detected by Lekowska Kochanial *et al.* (1996) (36%), however, Kalman *et al.* (2000) failed to detect plasmid DNA of C. jejuni isolate.

The relation between possession of plasmid DNA and the tested isolates and the antimicrobial resistance pattern showed that all the five strains that have plasmid showed a resistance against Cephalothin. Out of 5 isolates, 2 strains showed resistance against Flemukin and 3 out of 5 isolates showed resistance to Oxytetracycline. Table 6 summarized the antibiotic sensitivity of isolated Campylobacter jejuni. All the tested isolates were 100% sensitive to Gentamycin 10 mg, Nalidixic acid 30mg, Norfloxacin 10 mg, Enrofloxacin 5 mg. None of the isolates were sensitive to Cephalothin while, out of the tested isolates 60% were sensitive to Oxytetracycline 30 mg. Morever, 80% were moderately sensitive to Amoxacillin 30 mg while, 80% of isolates were weakly sensitive to Kanamycin. These results are nearly similar to those reported by Simor and Wilcax (1987) and Mouffok and Lebres (1992).

The antibiogram of C. jejuni isolated from milk and milk products often reflect the misuse of antibiotics in veterinary practice used in virtually all farms may have contributed to the resistance to some antibiotics.

Owing to the importance of C. jejuni as a food-bome pathogen, documented by various outbreaks and sporadic cases of human Campylobacteriosis, allover the world, the importance of carrying out a

prospective study on the prevalence of this pathogen in milk and some milk products is widely acknowledged.

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