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SURVEILLANCE OF *HELICOBACTER* SPECIES IN MILK AND SOME MILK PRODUCTS IN ASSIUT GOVERNORATE

(With 6 Tables and One Figure)

By

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مدى تواجد ميكروب الهيلكوباكتر فى اللبن وبعض منتجاته فى محافظة أسيوط

نجاح سعد ، إيناس البرنس

يعتبر ميكروب الهيلكوباكتر من الميكروبات التى تمثل خطوره على صحة المستهلك لما يسببه من إلتهاب وتقرح معدى ومعوى وفى النهاية يؤدى إلى سرطانات. من هنا كان الاهتمام بتحديد مدى تواجد هذا الميكروب فى الألبان ومنتجاتها من المطالب الملحة. لذا تضمنت هذه الدراسة جمع ١٢٥ عينة عشوائية من الألبان ومنتجاتها من المحلات الصغيرة والباعة الجائلين ومنازل الفلاحين فى محافظة أسيوط بواقع ٢٥ عينة من كل من اللبن الخام، لبن الغنم الخام ، جيلاتى ، جبن قريش وزبد فلاحى. أثبتت النتائج أن ميكروب الهيلكوباكتر تم عزله من ٤، ١٦ و ٢٠% من عينات اللبن الخام ، الجبن القريش والزبد الفلاحى التى تم فحصها على التوالى. من ناحية أخرى فقد كانت عينات لبن الغنم الخام والجيلاتى خالية من الميكروب ، وقد تم عزل أربعة أنواع من عترات الهيلكوباكتر من الألبان ومنتجاتها وهى ***H.pylori , H. felis, H. fennelliae*** and ***H. cinaedi*** وقد تم عـزل ميكـــروب ***H. pylori*** من ٤% من عينات كل من الجبن القريش والزبد الفلاحى فى المقابل تم عزل ميكروب H. felis من ١٢ و ٨% من الجبن القريش والزبد الفلاحى على التوالى ، ميكروب ***H. fennelliae*** من ٨% من الزبد الفلاحى بينما تم عزل ميكروب ***H. cinaedi*** من ٤% من عينات اللبن الخام. من بين منتجات الألبان كان الجبن القريش والزبد الفلاحى الأكثر تلوثا بميكروب الهيلكوباكتر. وباستخدام التحليل الالكتروفوريسى تبين أن خمسة من سبعة معزولات من ميكروب الهيلكوباكتر كانت تحمل بلازميدات تراوحت أحجامها الجزيئيه من ٢ –٤,٨ Mda وكذلك تم إجراء اختبارات الحساسية لميكروب ***H. pylori*** لعدد ١٨ من المضادات الحيوية. وأظهرت النتائج عن وجود مقاومة من الميكروب الذى يحمل بلازميد لستة من المضادات الحيوية بنسبة ٣٣,٣%. هذا وقد نوقشت الأهمية الصحية لميكروب الهيلكوباكتر والشروط الواجب إتباعها لمنع تلوث الألبان المختلفة ومنتجاتها بهذا الميكروب.

SUMMARY

One hundred and twenty five random samples of milk and some milk products including raw marketable milk, raw sheep milk, street vendors ice cream, Kareish cheese and cooking butter (25 each), were collected from dairy shops, groceries, street vendors and farmers houses in Assiut Governorate. These samples were examined for prevalence of ***Helicobacter*** species using two selective media: H. Pylori Special Peptone Agar (HPSPA) and Columbia blood base agar and typing of the obtained isolates by plasmid profile, as well as antibiotic sensitivity pattern of the isolated strains. The obtained results indicated that ***Helicobacter*** species was isolated from raw marketable milk, kareish cheese and cooking butter samples in percentages of 4, 16 and 20%, respectively. Most of the isolated strains 8 (6.4%) were recovered on Columbia agar while, only 2 (1.6%) were isolated on HPSPA. However, these organisms failed to be detected in raw sheep milk and ice cream samples. Concerning ***Helicobacter pylori***, it has been isolated from only one (4%) sample of both kareish cheese (on Columbia agar) and cooking butter (on HPSPA). In addition, other ***Helicobacter*** species were recovered from the examined samples as ***H. felis, H. fennelliae*** and ***H. cinaedi***. Whereas, 3 (12%) of kareish cheese and 2 (8%) of cooking butter contained ***H. felis***. 2 (8%) of cooking butter contained ***H. fennelliae***, while one (4%) of raw marketable milk samples contained ***H. cinaedi.*** Concerning plasmid profile, 5 strains out of 7 strains tested were found to carry plasmid of high molecular weight, these isolates showed resistance against some antimicrobial agents used for antimicrobial sensitivity pattern. The public health hazard of ***Helicobacter*** species and the sanitary measures for improving milk and milk products quality were recommended.

Key words: *Surveillance,* ***Helicobacter*** *species, Milk, Milk products, Plasmid profile, Antibiotic sensitivity.*

INTRODUCTION

Over the past 20 years, the genus ***Helicobacter*** evolved rapidly due to isolation of novel species from a wide range of animals and humans. The genus now includes at least 24 formally named species as well as numerous ***Helicobacters*** not formally named. Nineteen of these formally named ***Helicobacters*** are found in the intestinal tract of animals, eight in humans and two in birds (Akin *et al.* 1995; Andersen *et al.* 1996; Stolte *et al.* 1997 and Zhang *et al.* 1998).

Helicobacter pylori is the best known and the most important in terms of global impact on human disease. The bacterium is a spiral shaped and Gram-negative rods. It was first isolated in 1983 (Warren and Marshall 1983).

Originally, the organism was named Campylobacter pyloridis because it was structurally similar to other Campylobacter species. Campylobacter pyloridis was renamed C. pylori to fit in with names of other enteric pathogen. In 1989, it was finally named ***Helicobacter pylori*** based on functional and enzymatic properties. ***H. pylori*** is the most prevalent world's pathogenic bacterial infection in humans infecting approximately half of the world's population (Lambert *et al.* 1995). More than three–quarters of the population in the developing world are infected with the organism from an early age (Mendall & Northfield 1995 and Xia & Talley 1997), as the prevalence of infection is higher in developing countries than in developed ones depending on environmental and socioeconomic factors (Mégraud 1995 and Bardhan 1997).

Infection with ***H. pylori*** causes numerous medical conditions, including gastritis, up to 8% peptic ulcers, gastric carcinoma and mucosa associated lymphoid tissue tumors (Dixon 1994; Lee 1994 and Komoto *et al.* 1998). Also, the International Agency for Research on Cancers (1994) classified ***H. pylori*** as carcinogenic to human. Moreover, it causes more than 90% duodenal ulcers. The production of cytotoxin by ***H. pylori*** may be a significant factor known to affect ulcerogenesis. In addition, many pathogenic strains also produce a highly immunogenic cytotoxin-associated protein (Ching *et al.* 1996 and Weel *et al.* 1996). An association between ***H. pylori*** infection of stomach and coronary heart disease was observed by Patel *et al.* (1995) and Whincup *et al.* (1996).

The discovery of ***H. pylori*** increased interest in the range of other spiral bacteria in the stomach, but also in the other parts of the alimentary tract (Warren and Marshall 1983). In 1987, Dent *et al.* described the presence of a gastric bacterium ***H. heilmannii***. The gastritis observed with ***H. heilmannii*** infection tends to be less severe than that due to ***H. pylori***, but infection has been found in association with duodenal ulceration, gastric ulceration, gastric carcinoma and lymphoma (Morgner *et al.* 2000). The majority of patients are asymptomatic, however epigastric pain or discomfort, nausea, vomiting, anorexia, weight loss, diarrhea and occasionally gastrointestinal bleeding may occur.

In recent years, new ***Helicobacter*** spp. have been isolated from the liver of a wide variety of animals and have been associated with hepatic disease in immunocompromised and immunopatent patients (Mendez-Sanchez *et al.* 2001). Fox *et al.* (1995) were able to identify ***H. bilis, H. pullorum*** and ***H. rappini*** with no detection of ***H. pylori***. These bacteria are transmitted to human from animals. Many of ***Helicobacters*** can colonize the biliary tract of the liver and induce hepatitis and in some cases hepatic cancer or cause bacteraemia and systemic disease in immunocompromised hosts (Rudi *et al.* 1999 and Nilsson *et al.* 2000).

Proposed methods of infection include direct oral-oral transfer, indirect transfer via fecal-oral routes, zoonotic transmission from animals, and transmission via such vehicles as food and water (Kelly *et al.* 1994 and Vincent 1995). The bacterium remained active for more than 20 days in river water (Shahamat 1993). Begue *et al.* (1998) found a correlation between increased consumption of food from street vendors and ***H. pylori*** infection, suggesting that preparation of food in an unhygienic condition could serve as a mode of transmission.

H. pylori could survive in some foods such as some dairy products (Banwart 1979) and survived for up to 10 days in milk at 4 °C storage (Fan et al. 1998). ***H. pylori*** could still reisolated from inoculated cooled milk after six days in a density up to 10^3 CFU/ml. At room temperature (37 °C) the pathogen could be detected in milk for three to four days only (Bohmler *et al.* 1996). Dore *et al.* (2001) demonstrated ***H. pylori*** in 60% of sheep milk samples suggesting that sheep may be a natural host for ***H. pylori***. However, Turutoglu and Mudul (2002) could not isolate the organism from raw sheep milk. Whereas, Fujimura *et al.* (2002) cultured ***H. pylori*** from one raw cow's milk sample.

It is unlikely that milk and milk products act as a vehicle of transmission. Therefore, the aim of this study was to evaluate milk and some milk products for ***Helicobacter*** species in Assiut Governorate with special reference to ***H. pylori***. Also, the screenings of isolated species for plasmid profile as well as their antibiotic sensitivity pattern were estimated.

MATERIALS and METHODS

From April to August 2003, a total of 125 random samples of milk and some dairy products were aseptically collected from dairy shops, groceries, street vendors and farmers' houses in Assiut Governorate. These samples, including raw marketable milk, raw sheep milk, street vendors ice cream, kareish cheese and cooking butter (25

each), were transferred to the laboratory with a minimum of delay to be examined for the concerned microorgansims. Milk samples were examined by Storch test according to Lampert (1975), to detect heat-treated samples.

Enrichment procedure:

One ml of each milk sample or 1 g of each product was transferred to 10 ml of a selective enrichment broth "Helicobacter Pylori Special Peptone Broth" (HPSPB) which was supplemented with antibiotics [vancomycin (10 mg/liter), amphotericin B (5 mg/liter), cefsulodin (10 mg/liter), polymyxin B sulphate (31.000 IU/liter) and trimethoprim (40 mg/liter)] and calf serum as described by Stevenson *et al.* (2000). The inoculated broth was incubated under a microacrophilic gas mixture (6% O_2, 10% CO_2 and 84% N_2) using gas pack system at 37°C for 48h.

Isolation and identification of the isolated strains:

Loopful of the incubated broth was streaked onto plates of 2 selective media: H. Pylori Special Peptone Agar HPSPA (A) and Columbia blood base agar (B). Inoculated plates were incubated at 37°C for 4 days under the previous microaerophilic conditions. The suspected colonies were inoculated into slope of the same media for morphological and biochemical tests as estimated by Koneman *et al.* (1994), Shen *et al.* (1997) and Hua *et al.* (1999). The isolates were examined for catalase, nitrate, urease, hippurate and the susceptibility to cephalothin (30 µg/disc) and nalidixic acid (30 µg/disc) was also determined.

Extraction of plasmid DNA:

It has been carried out in the Molecular Biology and Genetic Engineering Research Center in Assiut University. A single colony of ***Helicobacter*** species was grown to saturation in 5 ml of L-broth at 37°C for 10 h (overnight) in a shaking bath and 2 ml of each culture were transferred to 1.5 ml Eppendorf tube for plasmid extraction by using the alkaline lysis procedure as described by Woodford *et al.* (1994).

Detection of plasmid DNA by agarose gel electrophoresis:

10 µl of the extracted plasmid DNA treated by Rnase enzyme were mixed with 10 µl of loading buffer (pH 8.3) and the aliquots were loaded onto horizontal 0.7% agarose gel system (BioRad, Richmond, USA). Then inoculated to gel tray, the electric field used as 75 mA for 2-3 h. The standard Marker was the isolated plasmids obtained from E.coli V 517 of molecular weight ranged from 1.4-35.8 Mda stained by 0.5 µg/ml ethidium bromide solution. Electrophoresis was carried out for 20-30 min and photographed by direct screen instant camera (Polaroid

DS.34) under UV transilluminator (TFX-20M, Vilber Lourmat–France). The molecular weights were determined by matching the electrophoretic mobility of both marker and isolated plasmid DNA (Birnboim and Doyle 1979).

Antimicrobial susceptibility testing for *H.pylori* :

All positive cultures for ***H. pylori*** growth were tested for their sensitivity and resistance patterns to 18 different antimicrobial agents by disc diffusion method (Hartzen *et al.* 1997). All plates were incubated for 72 h at 37 °C in a microaerophilic atmosphere. Storage and peruse conditions of antibiotic sensitivity were strictly in accordance with the manufacturer's instructions.

RESULTS

The obtained results are recorded in Tables 1-6 and Figure 1.

Table 1: Prevalence of ***Helicobacter*** species in the examined samples of milk and some dairy products.

Examined samples	No. of examined samples	Positive samples		Media Used			
				HPSPA (A)		Columbia (B)	
		No.	%	No.	%	No.	%
Raw marketable milk	25	1	4%			1	4%
Raw sheep milk	25	-	-				
Ice cream	25	-	-				
Kareish cheese	25	4	16%	1	4%	3	12%
Cooking butter	25	5	20%	1	4%	4	16%
Total	125	10	8%	2	1.6%	8	6.4%

Table 2: Frequency distribution of ***Helicobacter*** species isolated from the examined samples of milk and some dairy products.

Examined samples	Isolated strains		Media Used			
			HPSPA (A)		Columbia (B)	
	No./10	%	No./10	%	No./10	%
Raw marketable milk	1	10%			1	10%
Raw sheep milk	-	-				
Ice cream	-	-				
Kareish cheese	4	40%	1	10%	3	30%
Cooking butter	5	50%	1	10%	4	40%

Table 3: Surveillance of ***Helicobacter pylori*** in the examined samples of milk and some dairy products.

Examined samples	No. of examined samples	Positive samples		Media Used			
				HPSPA (A)		Columbia (B)	
		No.	%	No.	%	No.	%
Raw marketable milk	25						
Raw sheep milk	25						
Ice cream	25						
Kareish cheese	25	1	4%			1	4%
Cooking butter	25	1	4%	1	4%		
Total	125	2	1.6%	1	0.8%	1	0.8%

Table 4: Surveillance of other ***Helicobacter*** species in the examined samples of milk and some dairy products.

Examined Samples	Isolated strains					
	H. felis		*H. fennelliae*		*H. cinaedi*	
	No./25	%	No./25	%	No./25	%
Raw marketable milk					1 *(B)	4%
Raw sheep milk						
Ice cream						
Kareish cheese	2 *(B) 1 *(A)	8% 4%				
Cooking butter	2 *(B)	8%	2 *(B)	8%		

* (A): HPSPA * (B): Columbia agar base

Table 5: Correlation between isolates of ***Helicobacter*** species and plasmids.

Examined samples	No. of isolates	Species	Plasmid bearing		Not-bearing plasmid	
			No./7	%	No./7	%
Raw marketable milk	1	*H. cinaedi*	1	14.3%		
Kareish cheese	3	*H.felis* *H.pylori*	1 1	14.3% 14.3%		
Cooking butter	4	*H.pylori* *H.fennelliae* *H.felis*	1 1	14.3% 14.3%	2	28.5%
Total	7		5	71.5%	2	28.5%

Table 6: Antibiotic sensitivity pattern of plasmid bearing ***Helicobacter pylori.***

Types of antibiotics	Sensitive	Intermediate	Resistant
- Amoxycillin	+		
- Cephalosporin		+	
- Chloramphenicol			+
- Ciprofloxacin			+
- Colistin			+
- Danoflexalin	+		
- Enrofloxacin	+		
- Erythromycin			+
- Florfenicol	+		
- Flumequine	+		
- Gentamycin		+	
- Kanamycin			+
- Lincospectin			+
- Nalidixic acid			+
- Norflexacin		+	
- Oxalinic acid		+	
- Pefloxacin	+		
- Trimethoprime			+
Total	6 (33.3%)	4 (22.3%)	8 (44.4%)

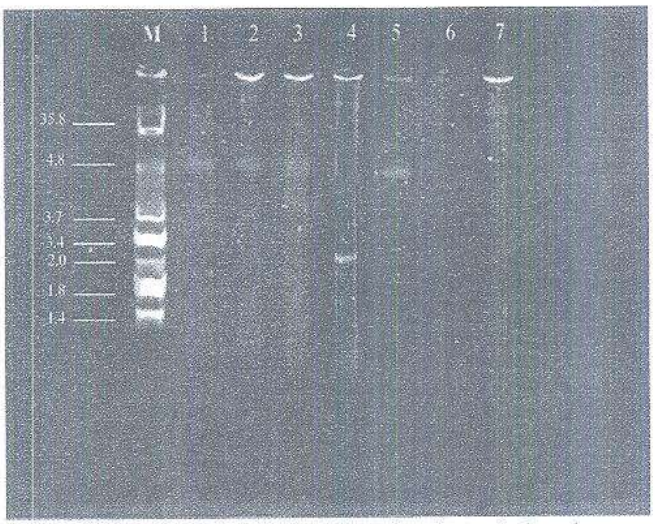

Fig. 1: Plasmid pattern of the isolated *H.* spp. from the examined samples.
- Lanes 1,2, 3 and 5 carry plasmid DNA (4.8 Mda).
- Lane 4 : carry plasmid DNA (2.0 Mda).
- Lanes 6 and 7 : not bearing plasmid DNA.

DISCUSSION

It is apparent from the results recorded in Table 1 that 8% of the examined raw milk and milk products were contaminated with ***Helicobacter*** spp. It could be observed that cooking butter (20%) followed by kareish cheese (16%) were the most contaminated samples, while raw milk samples were the least (4%). On the other side, raw sheep milk and ice cream samples were negative for ***Helicobacter*** spp.

During the course of this study, Table 2 showed the frequency distribution of ***Helicobacter*** spp. in the examined raw milk and milk products. Overall and out of the 125 samples examined, 10 (8%) samples were positive for ***Helicobacter*** spp.

Much of the recent work on ***Helicobacters*** concentrated with ***H. pylori*** because of the association of this bacterium with human gastrointestinal diseases, although new species of this genus are currently being recognized. As shown in Tables 3 and 4 *Helicobacters*

represented mainly by ***H. pylori, H. felis, H. fennelliae*** and ***H. cinaedi. H. pylori*** recovered only from kareish cheese and cooking butter samples. It was encountered in 4% of both samples tested.

In this study the presence of ***H. pylori*** in the examined kareish cheese and cooking butter samples suggests that contamination of milk and milk products with this organism within dairy industry by insufficient hygiene management of infected persons.

The obtained results are adequately consistent with those reported by Turutoglu and Mudul (2002) who could not detect ***H. pylori*** in any sample of the examined raw sheep milk. In contrary, Dore *et al.* (2001) could detect ***H. pylori*** from raw sheep milk.

There is a little information in the literature about the incidence of ***H. pylori*** in milk and milk products. In this study, ***H. pylori*** could not be isolated from the examined raw marketable milk samples. This finding is in general agreement with the result of Jiang and Doyle (2002) who could not detect ***H. pylori*** in 120 raw bovine milk samples. On the other hand, Fujimura *et al.* (2002) cultured ***H. pylori*** in one raw cow's milk sample so, there is a possibility that milk is a transmission vehicle in ***H. pylori*** infection.

In 1983, ***H. pylori*** was discovered and identified as the causative agent of chronic gastritis and duodenal ulcer disease (Warren and Marshall 1983). Since the first isolation of ***H. pylori*** from humans, a number of ***Helicobacters*** have been identified during the last decade in domestic and laboratory animals.

It was clear from the results in Table 4 that, ***H. felis***, was the most prevalent species encountered in the examined kareish cheese and cooking butter samples. ***H. fennelliae*** ranked second in the number of cases of isolation comprising 8% in the examined butter samples while, ***H. cinaedi*** was isolated only from 4% of the examined raw marketable milk samples.

As recorded in Tables 1 and 2 the numbers of ***Helicobacter*** spp. detected on Columbia agar base (B) were higher than that on HPSPA (A) in all samples examined.

The obtained results declared that there is a significant difference between the two used media (B) and (A) in the isolation of ***Helicobacter*** spp. So, for successful detection of ***Helicobacter*** spp., the use of two media is recommended.

A notable finding is that most of ***Helicobacter*** positive samples were from kareish cheese and cooking butter, which usually made from raw milk. This emphasized the role of primitive way of processing and

unhygienic handling of these particular types of dairy products as an important source of contamination with ***Helicobacters***.

Finally, we observed that of all of the samples examined, ***H. felis*** was the most prevalent organism. While this organism is not a problem from a regulatory point of view, control and elimination of this organism should eliminate the less frequently found pathogenic species ***"H. pylori"***.

The presence of any ***Helicobacter*** spp. should be a warning to dairy food producers that conditions exist for the growth of pathogenic spp. of ***Helicobacters*** and that corrective measures needed to ensure the production of pathogen-free foods.

Unfortunately owing to the ecological habitat of the organism, its eradication is not simply easy. Moreover, primary and acquired antimicrobial drug resistance has emerged as an important cause of treatment failure (Rozynek *et al.* 1995). As well as, ***H. pylori*** eradication is an important clinical goal in the treatment of infected patients with peptic ulcer disease and the ***H. pylori*** associated conditions (Hunt *et al.* 2000).

The data in Table 5 and Fig. 1 indicated that out of 7 tested strains, 5 were found to carry plasmids, lanes 1, 2, 3 and 5 where produced identical pattern of molecular weight at 4.8 Mda and lane 4 at 2.0 Mda, constituted 71.5%. These strains were belonged to ***H. cinaedi; H. felis*** & ***H. pylori*** and ***H. pylori*** & ***H. fennelliae*** each in a percentage of 14.3% which recovered from raw marketable milk; kareish cheese and cooking butter samples, respectively.

Penfold *et al.* (1988) at first reported 48% of clinical isolates of Campylobacter pylori to possess plasmids. While, Dharmalingam *et al.* (2003) recorded that isolates harbouring plasmids were seen in patients of peptic ulcer disease and matched control constituted 5.4% of total isolates. These findings were relatively lower than the obtained results in this study.

However, 2 strains (28.5%) of ***H. felis,*** isolated from cooking butter, not bearing plasmid DNA. Furthermore, ***H. pylori*** isolates carrying plasmid DNA were analysed for possible relationship between the presence of plasmids and their antimicrobial sensitivity pattern. The antimicrobial agents used were denoted in Table 6 in which the resistance of ***H. pylori*** were as follows: 6 (33.3%) sensitive, 4 (22.3%) intermediate and 8 (44.4%) resistant.

In vitro, antibiotic susceptibility testing for ***H. pylori*** should be done to guide the selection of antibiotic and predict the clinical response

to treatment for successful eradication (Smaill 2000). In many pathogenic bacteria, plasmids frequently carry antibiotic resistance encoding genes besides encoding virulence factors allowing bacteria to survive antibiotic treatment besides encoding virulence factors (Dharmalingam *et al.* 2003).

In conclusion, results obtained in this study reflect the importance of way of processing as well as the storage condition of dairy products on its final hygienic status, which may enhance or inhibit microbial contaminants present. So sailing of dairy products should be strictly controlled with health authority to eliminate potentiality of occurring hazards arising from microbial contamination.

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