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A STUDY ON *H. PYLORI AND H. HEILMANNII* IN MILK, SOFT CHEESE AND THE HEALTH HAZARD ON CHILDREN IN ASSIUT GOVERNORATE

(With 3 Tables and 3 Figures)

By

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دراسة عن *H. pylori* و *H. heilmannii* فى اللبن والجبن والخطورة الصحية على الأطفال فى محافظة أسيوط

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تعتبر ميكروبات الهيلكوباكتر من الميكروبات التى تمثل خطورة على صحة الإنسان والتي قد تؤدى إلى الإصابة بقرحة المعدة وفى بعض الاحيان قد يتطور الى حدوث سرطان فى المعدة. ولقد أجريت هذه الدراسة لمعرفة دور اللبن والجبن كمصدر لنقل ميكروبات الهيلكوباكتر الى الإنسان ، هذا بالإضافة إلى دراسة مدى تواجد كل من *H. heilmannii*. *H . pylori* فى الأطفال المصابين بالتهاب فى المعدة وقد تم عزل ميكروب *H. pylori* بنسبة 20% و6% من ألبان المزارع والألبان المعروضة للبيع فى أماكن مختلفة من محافظة أسيوط على التوالي. وقد تم عزل *H. heilmannii* من البان المزارع بنسبة 2% وتم عزل *H . pylori* و *H . heilmannii* بنسبة 26% و 6، 18% على التوالى من الأطفال. وقد تم مناقشة الأهمية الصحية لكل من *H . pylori* , *H . heilmannii* ومناقشة التوصيات لكيفية مقاومة ميكروبات الهيلكوباكتر.

SUMMARY

Helicobacter microorganisms are considered one of the important zoonotic microorganisms with public health hazard leading to gastric ulcer which may progress to gastric cancer. This study was designed to elucidate whether raw milk and soft cheese can act as a source of *Helicobacter*

infection to man. Moreover, investigation was carried out to determine the incidence rate of *H. pylori* and *H. heilmannii* among children suffering from gastric diseases. *H. pylori* was isolated from 20% and 6% of the examined milk from dairy farms and dairy shops, respectively. *H. heilmannii* was isolated from 2% of farm milk. *H. pylori* and *H. heilmannii* were isolated from 26% and 8 % of the examined children, respectively. In conclusion, milk may act as a vehicle of *H. pylori* and *H. heilmannii* and pose a health hazard to man. It is recommended to begin treatment early in patients with gastritis to accelerate healing and prevents ulcer production.

Key words: *H. pylori, H. heilmannii, milk, human, polymerase chain reaction, stool antigen.*

INTRODUCTION

Helicobacter species are wide spread micro-organisms and have been isolated from the gastric mucosa of a wide variety of wild and domestic mammals as well as humans (Gueneau *et al.,* 2002). Although transmission pathways of *Helicobacter* microorganisms to human are still unclear, several routes of transmission have been suggested (Allaker *et al.,* 2002). *Helicobacte*r microorganisms are transmitted from animals to man either through fecal oral transmission or consumption food of animal origin especially, milk and milk products (Van Duynhoven and de Jonge, 2001; Fujimura *et al.,* 2002; Roma-Giannikou *et al.,* 2002).

H. pylori is considered as the major agent of causing chronic gastritis worldwide and infects approximately one-half of the world's population (Everhart, 2000). *Helicobacter heilmannii,* has also been implicated as a potential cause of gastric disease in humans and rarely reported in children (Solnick *et al.,* 1993). Infection with *H. heilmannii* is thought to be considerably less frequent than infection with *H. pylori* (Fawcett *et al.,* 1999). *Helicobacter* infection in man may be asymptomatic (Parsonnet, 1998) however; it may cause chronic gastritis, gastric ulcer and duodenal ulcer (Ozturk, *et al.,* 1996). Infection with *Helicobacter sp*ecies are strongly linked to development of gastric carcinoma and gastric B-cell lymphoma (Suerbaum and Michetti, 2002).

There are several methods for detecting the presence of *Helicobacter* infection, each having its own advantages and disadvantages. Basically these tests are classified into two categories depending on whether endoscope biopsy is necessary. Histological evaluation, culture, polymerase chain reaction (PCR) and rapid urease tests are typically

performed on tissue obtained at endoscopy. Alternatively simple breath test, serology and stool assay are used without the need of biopsy (Bravos and Gilman. 2000).

This study was designed to elucidate the role of raw milk and soft cheese as a source of *Helicobacter* infection to man. On the other hand, investigation was carried out to determine the prevalence of *H. pylori* and *H. heilmannii* among children suffering from gastric diseases. The study was carried out in the period from May 2007 to April 2008.

MATERIALS and METHODS

Milk and cheese samples:

150 samples of raw milk from dairy farms, dairy shops and soft cheese (50 each) were collected randomly from different geographical regions in Assiut Governorate.

Children:

The study included 50 children (2-15years) with symptoms of persistence or recurrent upper abdominal discomfort including nausea, vomiting, haematemesis, heartburn and bloating or early satiety examined at gastroenterology unit of Assiut children university hospital. Informed consent of parents was obtained before enrollment in the study.

Exclusion criteria:

Children who had taken antimicrobials, antacids, H2 blockers, proton pump inhibitors, or bismuth sub salicylate within the 4weeks prior to endoscopy were excluded from the study. Two gastric biopsy samples were obtained from the antrum of each patient. One of the biopsy samples was fixed in 10% buffered formalin and embedded in paraffin for histopathological examination. The other biopsy was stored in normal saline on ice for bacteriological isolation.

Histological examination:

The paraffin embedded samples were cut at 5µg and the sections were stained with modified Giemsa and examined with light microscopy. All sections were examined to detect the grading of gastritis and the presence or absence of *H. pylori* and *H. heilmannii*. *H. heilmannii* is a spiral 7-10 µm (Oliva *et al.*, 1993) whereas, *H. pylori* is a small, curved bacteria 2-5 µm (Marshall, 1983). Bacterial distribution was assessed by the number of colonized crypts. Mild abundance is considered when, *Helicobacter* organisms found only in one crypt, moderate abundance is considered when, several crypts were colonized with the organisms and severe abundance is considered when both crypts and surface epithelium were colonized.

Bacteriological isolation:

Enrichment:

Gastric mucosal biopsy specimens were minced in saline with a sterile glass rod. A drop of the material was transferred to the selective enrichment broth for *Helicobacter*.

1ml of each milk sample or 1g of each cheese sample was transferred to selective enrichment broth for *Helicobacter* (*H. pylori* special peptone broth) which was supplemented with vancomycin 10mg /litre, amphotercin B sulphate 31.000 IU/ litre trimethoprim 40mg /litre and calf serum (Stevenson *et al.,* 2000). The inoculated broth was incubated under microaerophilic condition (6% O2, 10% CO2 and 84% N2) at 37˚C for 48h.

Isolation and Identification:

Loopful of the incubated broth was streaked onto plates of *H. pylori* special peptone agar media (HPSPA) and incubated at 37˚C for 4 days under microaerophilic condition (6% O2, 10% CO2 and 84%N2). Suspected colonies were maintained on HPSPA slants for further identification. Colonies were identified on the basis of their colony morphology, Gram staining and positive reaction with urease, catalase and oxidase (Fox *et al.,* 2000). Suspected colonies of *H. heilmannii* were maintained on HPSA slants for further identification with PCR.

DNA extraction and amplification:

DNA of the suspected colonies of *H. heilmannii* was extracted by using Guanidium thiocynate (Boom *et al.,* 1990). *H.heimannii* urease B gene was amplified by using primers

F 5´-GGGCGATAAAGTGCGCTTG-3´ and

R 5´CTGGTCAATGAGAGCAGG -3´ (Neiger *et al.,* 1998). Amplification was performed in a total volume of 25µl containing 2 µl of DNA extracts of each colony, 100pmol of each primer. The PCR was conducted in a Biometra Thermal cycler (Biometra – Germany) and the amplification condition was one cycle of 94˚C for 30 min, 72˚C for 3 min, followed by 31cycles of 94˚C for 30 sec, 57˚C for 3 sec, and 72˚C for 1 min and final extension at 72˚C for one min. PCR products (10µl) were analyzed by electrophoreses on 2% agarose gel containing ethidium bromide and visualized under UV illumination. PCR product size is 580 bp (Figure 1).

***H. pylori* stool antigen test:**

Stool sample of each patient was collected and transported directly to the lab and stored at -20^{o}C until processed. Qualitative enzyme linked immunosorbent assay (Astra s.r.l Via Ciro Menotti 1/A 20129 Milano) (www.astradiagnostici.com) was used to detect the presence of *H. pylori*

antigens in the stool. The protocol was followed according to manufacturer's procedure.

RESULTS

Table 1: Incidence of *Helicobacter* species in the examined samples

Helicobacter species	Source of Samples							
	Farm milk		Dairy shops		Cheese		Human	
	No.	%	No.	%	No.	%	No.	%
H. pylori	10	20	3	6	-	-	13	26
H. heilmannii	1	2	1	2	-	-	4	8
Total	11	22	4	8	-	-	17	34

Table 2: Detection of *H. pylori* in children by different methods

Bacterial isolation		Stool antigen		Histopathology	
No.	%	No.	%	No.	%
13	26	13	26	13	26

Table 3: Correlation between the endoscopic findings and the existence of *H. pylori* and *H. heilmannii*

Endoscope findings	No./50	*H. pylori*	*H. heilmannii*
Gastritis grade I	19	6(31.58%)	2(10.53%)
Gastritis grade II	6	2(33.33%)	1(16.67%)
Gastritis grade III	3	2(66.67%)	-
*Total	28	10(35.71%)	3(10.71%)
Gastric ulcer	1	1(100%)	-
Normal	21	2 (9.52%)	1(4.76%)

Gastritis grade I: some hyperemia in the gastric mucosa.
Gastritis grade II: Mosiac hyperemia in the gastric mucosa.
Gastritis grade III: Coublestone appearance of gastric mucosa.
*chronic active gastritis: grade I, grade II, grade III

Figure1: PCR identification of *H. heilmannii*
M marker, lanes 1, 3, 4, 5, 6, 7 *H. heilmannii* isolated strains lanes: 2, 8 negative samples.
Human strains 1,3,4,5. Milk strains:6, 7.

Figure : 2 Giemsa stained *H. pylori* organisms in gasrtric biopsy

Figure : 3 Giemsa stained *H. heilmannii* organisms in gasrtric biopsy

DISCUSSION

Epidemiological investigation revealed that food especially of animal origin play an important role in transmission of *Helicobacter* infection to man (Gomes and De Martinis, 2004). Although milk is not likely to contain *Helicobacter* organisms, it is considered as a source of infection after external contamination from the surrounding environment (Bohmeler *et al.,* 1996). *Helicobacter* species were isolated from 22% of milk samples obtained from dairy farms and 8% of milk samples obtained from dairy shops (Table 1). We could not isolate *Helicobacter* species from cheese. *H. pylori* was isolated from milk of farms and dairy shops with a rate of 20% and 6%, respectively (Table 1). Higher prevalence rates (72.2%) and (50%) of *H. pylori* were reported in cow's milk, respectively (Fujimura *et al.,* 2002; Quaglia *et al.,* 2008). Several investigators had incriminated milk as a vehicle of *H. pylori* transmission (Fan *et al.,* 1998; Fujimura *et al.,* 2002 & Van Duynhoven and de Jonge, 2001). It has been reported that *H. pylori* can survive in milk for up to 10 days at 4°C storage (Fan *et al.,* 1998). However, other studies failed to detect *H. pylori* in milk (Poms and Tatini 2001; Jiang and Doyle, 2002).

H. heilmannii was isolated from 2% of milk from dairy farms and dairy shops (Table 1). Although no data is available in the literature about the isolation of *H. heilmannii* from milk, our result is not unpredictable as once contamination of milk occur, *H. heilmannii* can be detected. It has been suggested that *H. heilmannii* infection in human is a zoonosis and

animals serve as reservoirs for transmission to humans (Meining *et al.,* 1998*).*

Helicobacter species were isolated from 17(34%) of the examined human biopsy with a rate of 26% for *H. pylori* and 8 % for *H. heilmannii* (Table 1). The prevalence rate of *H. pylori* and *H. heilmannii* obtained in this study among the examined children is considered within the range reported in the developing world and the previous research reported in Egypt (Bassily *et al.,* 1999; Naficy *et al.,* 2000). Higher prevalence rates of *H. pylori* (48% and 46%) were reported, respectively (Clemens *et al.,* 1996; Sherif *et al.,* 2004). However, lower prevalence rate (8.9%) was reported in another study (Karine *et al.,* 1999).

Concerning *H. heilmannii* isolation, lower prevalence rate (0.4%) was obtained previously (Karine *et al.,* 1999). It has been also reported that the prevalence rate of *H. heilmannii* in humans varies from 0.5% to 6% (Wooten *et al.,* 2004). Moreover, it has been reported that *H. heilmannii* accounts for 0.2% to 0.4% of human gastritis cases (Solnick *et al.,* 1993). On the other hand it has been reported that the frequency of *H. heilmannii* infection ranged from 0.25% to 1.2%-1.7% (Heilman and Borchard, 1991; Kubonovak *et al.,* 1994; Yang *et al.,* 1995). The variable rates of *Helicobacter* isolation in different studies vary greatly, depending on the location of study group and the characteristics of the population studied. Generally, prevalence of *Helicobacter* infection correlates positively with low socioeconomic status and increases with age (Malaty and Graham 1994).

On histopathological examination of the biopsy samples, 10(35.71%) and 1(100%) of the samples showed *H. pylori* associated with chronic active gastritis and gastric ulcer, respectively (Table 3). On the other hand 3 (10.71%) of biopsy samples showed long spiral organisms corresponding to *H. heilmannii* and were associated with chronic active gastritis (Table 3). *Heilcobacter* organisms were demonstrated either in the mucus or on the cell surface (Figure: 2 and 3). Although *Helicobacter* species were isolated from patients with different grades of gastritis on endoscopy, it has been also isolated from patients, with normal gastric mucosa with a percentage of 9.52% for *H. pylori* and 4.76% for *H. heilmannii* (Table 3). Our findings are in concurrence with that previously reported (Hassall and Dimmick 1991). *H. pylori* infection like other bacteria can induce an inflammatory process in the tissue. The organism is found in the lamina propria that indicates the organisms invade the gastric mucosa and this can explain why the organism escapes from eradication. There are some mediators that help in inflammation like IL2, IL7 and TNF-oxidative radicals by monocytes and macrophages (Glassman 1992).

Histopathological examination of the biopsy samples reveals that colonization of *H. pylori* was detected with moderate to severe density on the surface epithelium and within the crypts. In contrast *H. heilmannii* was detected with mild to moderate density in one crypt or several crypts, but no colonization of the surface epithelium was observed. Our result is in concurrence with that previously reported (Karine et al., 1999).Our data show that the patchy distribution of *H. heilmannii* was found in the antrum and this result is concurrent with another study (Karine *et al.,* 1999). Moreover, it has been described that the distribution of *H. heilmannii* was more frequently in the antrum in contrast to *H. pylori,* which was often present in both the antrum and fundus (Wooten *et al.,* 2004). Neither atrophy nor metaplasia was observed in the examined biopsy samples of *H. pylori* and *H. heilmannii.* Similar results were described in the literature (Heilmann and Borchard 1991; Oliva *et al.,* 1993).

Although histopathology is considered the most reliable method to diagnose *Helicobacter* infection (Drumm *et al.,* 2000; Gold *et al.,* 2000), stool antigen test was accepted by clinical researchers and by community physicians as it provides an easy method for epidemiological investigation as well as for clinical diagnosis (Elitsur, 2005). In our study we could detect the same infection rate (26%) by using different methods, including bacterial isolation, histopathology and stool antigen test (Table 2).

In conclusion, results obtained in this study reveal that milk may act as a vehicle of *H. pylori* and *H. heilmannii* which pose a public health hazard. Careful sanitary measures coupled with personal hygiene should be applied in farms as well as during milk processing to improve the microbiological safety of milk and milk products. Diagnosis of *H. heilmannii* infection are usually difficult because of its sparseness and patchy distribution so, we suggest that it is important to look carefully for *H. heilmannii* at histological examination especially in the cases of *H. pylori* negative gastritis in children. It is recommended to begin treatment early to accelerate healing and prevents long term ulcer production. Therapy should include a gastric acid production blocker (histamine h2 blocker or proton- pump inhibitor) together with two antibiotics (sulfamethoxazole and amoxicillin) for at least 3 months.

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