# Prevalence of *Helicobacter pylori* in Iranian Milk and Dairy Products using Culture and *ureC* based-PCR Techniques

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## ABSTRACT

The hygienic quality of milk has an important public health importance. *Helicobacter pylori* has a foodborne route and especially food with animal origin. This present study was carried out in order to isolation the *H . pylori* from cow milk samples using culture and PCR method. A total of 120 bovine milk and 120 dairy product samples were collected from the supermarkets of various parts of Iran. The animals whose milk samples collected for this study were clinically healthy.All samples were cultured and those that were *H. pylori*-positive were subjected for PCR method fordetection of the *H. pyloriure C* gene.Of 240 samples studied, 33 (13.75%) were positive for the *H. pylori* using the culture method. Raw bovine milk were the most contaminated (16.66%) but traditional cream were the less contaminated (7.5%) samples. Significant differences was seen for the prevalence of *H. pylori* between raw bovine milk and traditional cream samples (*P* =0.027). All of the positive colonies of *H. pylori* were confirmed using the *ureC* gene based-PCR method. This study showed that cow milk and traditional dairy product samples are the sources of *H. pylori* infection for humans.

**Key words:** *Helicobacter pylori*, Isolation, Cow milk, Culture, Polymerase Chain Reaction.

## INTRODUCTION

Milk is raised as a complete food especially for children and seniors. Its high value for proteins, minerals, fats and vitamins is undeniable and in a day, millions of people use the milk and dairy products. Therefore, hygienic quality of milk has a high importance in public health but sometimes it will be changed and several infections and illness are occurred.

The *Helicobacter pylori*(*H. pylori*) is a microaerophilic Gram negative bacterium with curved spiral shape which known as a causative agent of type B gastritis, peptic ulcer disease, gastric adenocarcinoma and Mucosa Associated Lymphoid Tissue (MALT) lymphoma[1].The bacterium has been classified as a Class I carcinogen by the World Health Organization[2].The worldwide prevalence of infection has a vast range from 40% to 80% [3, 4].The main protocol for treatment of diseases caused by *H. pylori*, is antibiotic therapy but the antibiotic therapy fails in about 20% of the patients[4], mainly due to antibiotic resistance[5]. During the last two decades, the role of *H. pylori* as potential pathogens in both human and veterinary

medicine has been investigated intensively, and evidence suggests possible zoonotic transmission of animal helicobacter to humans.

Therefore, accurate, sensitive and rapid detection of *H. pylori* in samples with animal origin plays an important role in control of diseases. There are various methods for diagnosis of brucellosis such as culture, serological and molecular methods. Culture methods are well established for *H. pylori* and there is a study which confirmed its application[6]. The diagnosis of *H. pylori* by serological responses, which can be unspecific due to cross-reaction or sub-sensitive reactions in samples from areas with a low or subclinical prevalence of helicobacteriosis[7]. Several studies showed that the molecular diagnosis of *H. pylori* is an applied form of its diagnosis. Several Polymerase Chain Reaction (PCR) methods have been developed for rapid, sensitive and accurate diagnosis of *H. pylori* in clinical samples[8-10].

This present study was carried out in order to detection the *H. pylori* in bovine milk samples using culture and PCR techniques in Isfahan, Iran.

## MATERIALS AND METHODS

### Samples

A total of 120 bovine milk samples and 120 traditional dairy products including cheese (n=80) and cream (n=40) were randomly collected from various parts of Iran. Samples were collected from 24 randomly selected dairy herds at spring of 2012. The animals selected for this study were clinically healthy and the milk samples showed normal physical characteristics. Samples were collected under sterile hygienic conditions and were immediately transported at 4°C to laboratory in a cooler with ice packs. All milk samples were kept at –20°C until processing.

### Isolation of H. pylori

Twenty five milliliter of each sample were added to 225 mL of Wilkins Chalgren anaerobe broth (Oxoid, UK) supplemented with 5% of horse serum (Sigma, St.

Louis, MO, USA) and colistin methane sulfonate (30 mg/L), cycloheximide (100 mg/L),

nalidixic acid (30 mg/L), trimethoprim (30 mg/L), and vancomycin (10 mg/L) (Sigma, St.

Louis, MO, USA) and incubated for 7 days at 37°C with shaking under microaerophilic conditions. Then, 0.1 mL of the enrichment selective broth was plated onto Wilkins Chalgren anaerobe agar (Oxoid, UK) supplemented with 5% of defibrinated horse blood and 30 mg/L colistinmethanesulfonate, 100 mg/L cycloheximide, 30 mg/L nalidixic acid, 30 mg/L trimethoprim, and 10 mg/L vancomycin (Sigma, St.

Louis, MO, USA)[6] and incubated for 7 days at 37°C under microaerophilic conditions. Suspected colonies were identified as *H. pylori* on the basis of the morphology of the colonies, Gram staining, and oxidase, catalase, and urease production[11]. The isolates were identified as *H. pylori* by using conventional bacteriological methods, were also positive using the PCR assay. For comparison, a reference strain of *H. pylori* (ATCC 43504) was employed.

### Detection of H. pylori using PCR method

DNA from 1 mL of each milk samples was extracted by a DNA isolation kit for cells and tissues (Roche Applied Science, Germany, 11814770001) according to the manufacturer's instructions and its density was assessed by optic densitometry. Extracted genomic DNA was amplified for the *ureC* gene and detected with the specific primers HP-F: 5'-GAATAAGCTTTTAGGGGTGTTAGGGG-3', HP-R: 5'GCTTACTTTCTAACACTAACGCGC-3'. The gene product was 294 bp. PCR reactions were performed in a final volume of 50 µL containing 5 µL 10 × buffer + $MgCl_2$, 2 mMdNTP, 2 unit Taq DNA polymerase, 100 ng genomic DNA as a template, and 25 picomole of each primer. PCR was performed using a thermal cycler (Eppendorf Co., Germany) under the following conditions: an initial denaturation for 10 minutes at 94°C; 35 cycles for 1 minute at 94°C, 1 minute at 55°C, 1 minute at 72°C, and a final extension at 72°C for 10 minutes. The PCR products were electrophoresed through a 1.5% agarose gels (Fermentas, Germany) containing ethidium bromide. A DNA ladder (Fermentas Co., Germany) used to detect the molecular weight of observed bands under a UV lamp. All tests were performed in triplicate. Samples

inoculated with *H. pylori* were used as positive controls.

### Statistical analysis

Data were transferred to Microsoft Excel spreadsheet (Microsoft Corp., Redmond, WA, USA) for analysis. Using SPSS 16.0 statistical software (SPSS Inc., Chicago, IL, USA), Chi-square test and Fisher's exact two-tailed test analysis was performed and differences were considered significant at values of *p*< 0.05.

### RESULTS

A total of 240 raw milk and traditional dairy product samples were analyzed for the presence of *H. pylori* using culture and PCR technique. All samples were cultured immediately. Table 1 shows the total distribution *of H. pylori* in various types of raw milk and dairy product samples. Results showed that 33 out of 120 milk and dairy products (13.75%) were positive for the *H. pylori* using the culture method. Raw bovine milk were the most contaminated (16.66%) but traditional cream were the less contaminated (7.5%) samples. Significant differences was seen for the prevalence of *H. pylori* between raw bovine milk and traditional cream samples (*P* =0.027). All of the positive colonies of *H. pylori* were confirmed using the *ureC* gene based-PCR method. Figure 1 shows the results of the gel electrophoresis for PCR products of the *ureC* gene of the *Helicobacter pylori* isolates from raw milk and dairy products. The results of the culture technique were also confirmed by the PCR method. No significant differences in detection of *H. pylori* in milk and dairy samples were observed between culture and PCR techniques.

### DISCUSSION

The results of the present study showed that the Iranian raw bovine milk and traditional dairy product samples were contaminated with *H. pylori*. Total prevalence rate of *H. pylori* in the samples of our investigation was 13.75% which was considerable high. One possible explanation for the high prevalence of *H. pylori* in our study is maybe that the animals which their milk samples were collected for this study were infected with *H. pylori*. Another reason is the fact that the workers of and stuffs of the traditional companies of dairy production were maybe infected with *H. pylori* and transmit this pathogen to these products. Infection of the water sources used for washing of boxes, dishes and containers of milk and dairy preparation
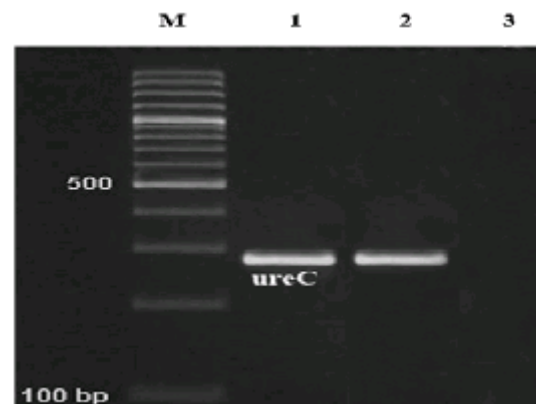


**Fig. 1: Results of the gel electrophoresis for PCR products of the ureC gene of the *Helicobacter pylori* isolates from raw milk and dairy products. M is 100bp DNA marker, 1 is positive sample, 2 is positive control and 3 is negative control**

**Table 1: Distribution *of Helicobacter pylori* in various types of raw milk and dairy product samples**

| Types of samples | No. samples collected | Positive results for *H. pylori* in culture (%) | PCR confirmation (%) |
|---|---|---|---|
| Bovine milk | 120 | 20 (16.66) | 20 (16.66) |
| Traditional cheese | 80 | 10 (12.5) | 10 (12.5) |
| Traditional cream | 40 | 3 (7.5) | 3 (7.5) |
| Total | 240 | 33 (13.75) | 33 (13.75) |

maybe another sources of high prevalence of *H. pylori*.

As far as we know, the *H. pylori* has been detected in various types of foods including milk[9], meat[12] and ready to eat foods[13].Previous study from Iran showed thatthree of 447 milk samples (0.67%), including two sheep (2.2%) and one buffalo (1.6%) milk samples, were found to be contaminated with *H. pylori*using the culture method while the *H. pyloriureC* gene was detected in 56 (12.5%) of milk samples, including 19 cow (14.1%), 11 sheep (12.2%), nine goat (8.7%), two camel (3.6%), and 15 buffalo (23.4%) milk samples using the PCR method[9] was entirely lower that Japan (72.2%)[14] andIran (previous study) (14.1%)[9]. Also, previous study from Iran showed that 16% of raw milk samples were contaminated with *H. pylori*[15]. In central Iran, the prevalence of *H. pylori* is very high (78%)[15]. One of the major sources of infection in humans could be cow, sheep, and goat's milk contaminated with *H. pylori*[16]. The consumption of milk and its products vary considerably in different regions in the world. Bovine milk and dairy products have a long tradition in human nutrition. Iranian people especially children and senior drink cow's milk commonly.Therefore, consumption of raw milk

has been considered as the main source for human helicobacter infection. Also, the *H. pylori* has been isolated from pasteurized milk samples, previously (55% prevalence rate)[14].The Greek study showed that the prevalence of *H. pylori* in cow's milk was 20% which was higher than our percentage[17].The study in USA showed that 60% of sheep milk samples were contaminated with *H. pylori*[18]. Funnily, the results of our study are similar with the results of Azevedo *et al.* (2007) which could not prove the existence of *H. pylori* in milk through culturing. These findings are very important to explain the way of transmission of *H. pylori* to humans through milk and food[19].

## CONCLUSION

All of the above studies from Iran showed that the *H. pylori* is endemic in this country and it is essential to use from well-boiled milk and traditional dairies for human consumption. It is tempting to speculate that cow were the ancestral host of the *H. pylori* and that it entered the human population after domestication of cow. It will be of interest to examine cows and dairy products in different regions to test the hypothesis that cows are natural hosts of *H. pylori*.

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