Genotyping of the *Helicobacter pylori* isolates of raw milk and traditional dairy products

Leila Khaji,[1] Gholamreza Banisharif,[2] Iman Alavi[2]

[1]Department of Veterinary Medicine, Faculty of Veterinary Medicine, University of Tehran; [2]Department of Microbiology, Faculty of Basic Sciences, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran

Abstract

Notwithstanding the substantial clinical impact of *Helicobacter pylori*, its convinced routes of transmission and sources have not been reported. Based on the quarrelsome hypothesis, foods and especially dairy products play an authoritative role in the transmission of *H. pylori* to humans. The current investigation was done to study the prevalence rate and distribution of *vacA* genotypes in the *H. pylori* strains isolated from the raw milk and traditional dairy products. Three-hundred milk and dairy samples were collected and directly transported to laboratory. Samples were cultured and *H. pylori* isolates were approved using the *16s rRNA*-based PCR amplification. Positive strains were tested for distribution of *vacA* genotypes using the multiplex-PCR. Sixty out of 300 samples (20%) harbored *H. pylori*. Prevalence of *H. pylori* in milk and traditional dairy products were 38.75% and 13.18%, respectively. Ovine milk (45%) and traditional cheese (40%) had the highest prevalence of *H. pylori*. *VacAs1a* (91.66%), *vacAm1a* (61.61%) *vacAs2* (36.66%) and *vacAm2* (31.66%) were the most commonly detected genotypes. Ovine milk and traditional cheese had the most diverse genotypes. *S1am1a* (41.66%), *s2m1a* (25%), *s1am2* (16.66%) and *s2m2* (13.33%) were the most commonly detected combined genotypes. Raw milk and traditional dairy products are latent sources of *H. pylori*. Similarity in the genotyping pattern of *H. pylori* strains of various samples represents their similar sources of infection. Further studies are required to found the exact sources of *H. pylori* strains in raw milk and traditional dairy products.

Introduction

The contamination of food with microbes can happen at any step of the food chain. Milk is virtually sterile when it is synthesized in a healthy animal's udder. Ruminants are natural reservoirs of bacteria. Many of these bacteria are not harmful to humans, but some may be harmful to humans even though the animals are not affected and appear healthy. The extremely nourishing nature of dairy products makes them particularly suitable media for bacterial proliferation. Raw milk and dairy products can harbor a variety of pathogenic organisms.[1]

Though *Helicobacter pylori* (*H. pylori*) have been considered as a main cause of mucosa associated lymphoma, peptic ulcer disease, type B gastritis, and gastric adenocarcinoma, roles of foods and especially dairy products in its transmission are still unknown.[2-4] *H. pylori* was found in stomach of domestic animals in the absence of associated gastritis, it was recovery from milk, meat and gastric tissue of domestic ruminants suggest that domestic animals and their milk may be reservoirs of *H. pylori*.[2-4] Suitable circumstances for growth and survival of *H. pylori* in milk and dairy products provide adequate settings for transmission of *H. pylori* from these foodstuffs to human.[5]

The severity of clinical complications caused by *H. pylori* is depends on several factors. Presence of pathogenic virulence genes and genotypes the most important factor responsible for *H. pylori* infections. Vacuolating cytotoxin (*vacA*) is one of the most important virulence factors in the occurrence of human clinical diseases caused by this bacterium. The *vacA* belongs to the group of genes with variable genotypes or structures. The *vacA* gene is present in virtually all strains of *H. pylori* but it is polymorphic, comprising variable signal regions (type *s1* or *s2*) and mid-regions (type *m1* or *m2*). The *s*1 type is additional subtyped into *s1a*, *s1b* and *s1c* subtypes, and the *m1* into *m1a* and *m1b* subtypes.[6,7] The mosaic combination of s and m-region allelic types determines the particular cytotoxin and, consequently, the pathogenicity of the bacterium. Genotyping using *vacA* virulence marker is considered as one of the best approaches for study of correlations between *H. pylori* isolates from different samples.[6,7]

Materials and Methods

Sample collection

From May 2015 to July 2015, overall 300 dairy products including raw bovine milk (n=40), raw ovine milk (n=40), traditional cheese (n=50), traditional cream (n=50), traditional butter (n=60) and traditional ice-cream (n=60) were purchased from the supermarkets of Tehran province, Iran. Samples (100 mL, in sterile glass containers) were transported to the laboratory at 4°C. All samples were kept under refrigeration in plastic bags; information about dates of production and of assigned shelf lives was not presented.

Isolation of *Helicobacter pylori*

Twenty five milliliters of each homogenized sample were added to 225 mL of Wilkins Chalgren anaerobe broth (Oxoid, UK) supplemented with colistin methanesulfonate (30 mg/L) and 5% of horse serum (Sigma, St. Louis, MO, USA) and nalidixic acid (30 mg/L), vancomycin (10 mg/L) cycloheximide (100 mg/L) and trimethoprim (30 mg/L) (Sigma, 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 colistin methanesulfonate, 100 mg/L cycloheximide, 30 mg/L nalidixic acid, 30 mg/L trimethoprim, and 10 mg/L vancomycin (Sigma, USA) and incubated for 7 days at 37°C under microaerophilic conditions. For

Correspondence: Leila Khaji, Doctor Veterinary Medicine, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.
Tel.: +98.9125.252383.
E-mail: L.kh52834@yahoo.com

Key words: *Helicobacter pylori*, Prevalence, Genotyping, Raw milk, Traditional dairy.

Acknowledgements: the authors would like to thank from Prof. Ebrahim Rahimi at the Department of Food Hygiene and Quality Control, Faculty of Veterinary Medicine, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran for his significant technical provision.

Contributions: the authors contributed equally.

Conflict of interest: the authors declare no potential conflict of interest.

Received for publication: 29 June 2017.
Revision received: 9 August 2017.
Accepted for publication: 19 August 2017.

Microbiology Research 2017; 8:7288
doi:10.4081/mr.2017.7288

comparison, a reference strain of *H. pylori* (ATCC 43504) was employed.

DNA extraction and *Helicobacter pylori* 16S rRNA gene amplification

Suspected colonies were identified as *H. pylori* based on the PCR technique. Genomic DNA was extracted from the colonies with typical characters of *H. pylori* using a DNA extraction 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 DNA was amplified for the *16S rRNA* gene (primers: HP-F: 5'-CTGGAGAGACTAAGCCCTCC-3' and HP-R: 5'-ATTACTGACGCTGATTGTGC-3') (110 bp).[8] PCR reactions were performed in a final volume of 50 µL containing 5 µL 10 × buffer + $MgCl_2$, 2 mM dNTP, 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 2 minutes at 94°C; 30 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s and a final extension at 72°C for 8 min.

Genotyping of vacA genes of *Helicobacter pylori*

Presence of the *vacA* (*s1a*, *s1b*, *s1c*, *m1a*, *m1b* and *m2*) alleles were determined using PCR technique. Primers, PCR conditions and volume of each reaction are shown in Table 1.[9] PCR amplifications were performed using a DNA thermal cycler (Eppendorf Co., Germany). All runs included one negative DNA control consisting of PCR grade water and two or more positive controls (26695, J99, SS1, Tx30, 88-23 and 84-183).

Gel electrophoresis

The PCR amplification products (10 µL) were subjected to electrophoresis in a 1.5% agarose gel in 1X TBE buffer at 80 V for 30 min, stained with ethidium bromide, and images were obtained in a UVIdoc gel documentation systems (UK). The PCR products were identified by 100 bp DNA size marker (Fermentas, Germany).

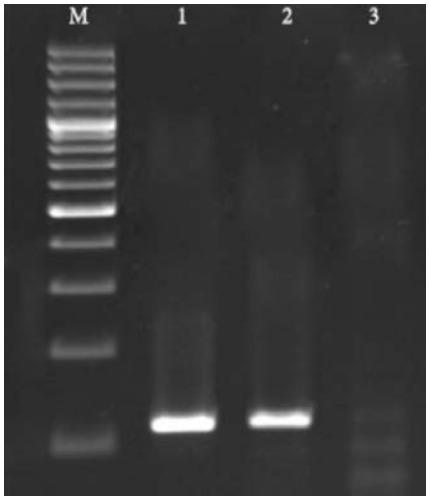

Figure 1. Results of the gel electrophoresis for the *16SrRNA* gene of the *H. pylori* strains isolated from dairy products. M: 100 bp ladder, 1: Positive sample for the *16SrRNA* gene (110 bp), 2: Positive control (*H. pylori* ATCC 43504) and 3: Negative control (PCR grade water (Fermetas, Germany)).

Table 1. Oligonucleotide primers and PCR conditions used for genotyping of *Helicobacter pylori* strains isolated from dairy products.[9]

Genes	Primer Sequence (5'-3')	Size of product (bp)	Volume of PCR reaction (50 µL)	PCR programs
vacA s1a	F: CTCTCGCTTTAGTAGGAGC	213	5 µL PCR buffer 10X (Fermentas)	1 cycle:
	R: CTGCTTGAATGCGCCAAAC		1.5 mM $Mgcl_2$	95 ^{0C} ---------- 1 min.
vacA s1b	F: AGCGCCATACCGCAAGAG	187	200 µM dNTP (Fermentas)	32 cycle:
	CTGCTTGAATGCGCCAAAC		0.5 µM of each primers F & R	95 ^{0C} ---------- 45 s
vacA s1c	F: CTCTCGCTTTAGTGGGGYT	213	1.25 U Taq DNA polymerase (Fermentas)	64 ^{0C} ---------- 50 s
	R: CTGCTTGAATGCGCCAAAC		2.5 µL DNA template	72 ^{0C} ---------- 70 s
vacA s2	F: GCTAACACGCCAAATGATCC			1 cycle:
	R: CTGCTTGAATGCGCCAAAC	199		72 ^{0C} ---------- 5 min
vacA m1A	F: GGTCAAAATGCGGTCATGG			
	R: CCATTGGTACCTGTAGAAAC	290		
vacA m1B	F: GGCCCCAATGCAGTCATGGA			
	R: GCTGTTAGTGCCTAAAGAAGCAT	291		
vacA m_2	F: GGAGCCCCAGGAAACATTG			
	R: CATAACTAGCGCCTTGCA	352		

Table 2. Prevalence of *Helicobacter pylori* in various dairy products.

Samples	No. samples collected	No. positive samples for *H. pylori* (%)	PCR confirmation (%)
Raw milk			
Bovine	40	13 (32.50)	13 (32.50)
Ovine	40	18 (45)	18 (45)
Total	80	31 (38.75)	31 (38.75)
Traditional dairy products			
Cheese	50	20 (40)	20 (40)
Cream	50	2 (4)	2 (4)
Butter	60	2 (3.33)	2 (3.33)
Ice-cream	60	5 (8.33)	5 (8.33)
Total	220	29 (13.18)	29 (13.18)
Total	300	60 (20)	60 (20)

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. Distribution of *H. pylori* genotypes isolated from dairy products were statistically analyzed.

Results

Table 2 represents the total prevalence of *H. pylori* isolated from different types of dairy products. All of the *H. pylori* isolates harbored the *16SrRNA* gene in the PCR amplification (Figure 1). We found that 60 out of 300 samples (20%) were positive for the *H. pylori*. Prevalence of *H. pylori* among milk and traditional dairy products were 38.75% and 13.18%, respectively. Ovine milk (45%) and traditional cheese (40%) had the highest prevalence of *H. pylori* strains. Statistically significant differences were seen between the type of samples and prevalence of *H. pylori* strains (P<0.05).

Table 3 represents the distribution of *vacA* genotypes among *H. pylori* isolates of dairy products. *VacAs1a* (91.66%), *m1a* (61.61%) *s2* (36.66%) and *m2* (31.66%) were the most commonly detected genotypes amongst the *H. pylori* isolates. Ovine milk and traditional cheese had the most diverse genotypes. *VacAs1c* (5%) had the lowest prevalence amongst the *H. pylori* isolates of all dairy samples. Statistically significant differences were seen between the type of samples and prevalence of different genotypes (P<0.05).

Table 4 represents the distribution of combined genotypes of the *H. pylori* strains isolated from all studied samples. We found that *s1am1a* (41.66%), *s2m1a* (25%), *s1am2* (16.66%) and *s2m2* (13.33%) were the most commonly detected genotypes among the *H. pylori* isolates of all dairy products. There were no positive results for *s1cm1b* and *s1cm2* combined genotypes.

Discussion

Results of the present study revealed that 20% of dairy products were contaminated with *H. pylori* strains which showed an important public health threat regarding the consumption of raw milk and traditional dairy products. Results also indicated the high distribution of putative genotypes in the *H. pylori* isolates of milk and dairy products.

Prevalence of *H. pylori* in dairy products of our study (20%) was higher than that of Rahimi and Kheirabadi (2012)[10] (Iran, 0.67% in milk samples), Gilani *et al.* (2017)[11] (Iran, 5% in meat samples) and Atapoor *et al.* (2014)[12] (9.56% in vegetable), Talaei *et al.* (2015)[13] (Iran, 4.76-20% in milk samples), Ghasemian Safaei et al. (2011)[14] (Iran, 16% in milk samples), *Esmaeiligoudarzi et al.* (2015)[15] (Iran, 13.75% in milk samples and dairy products), Mousavi *et al.* (2014)[16] (Iran, 19.80% in milk samples and 19.20% in dairy products) and Yahaghi *et al.* (2014)[9] (Iran, 14% in salad and 13.68% in vegetable), while was lower than that of Fujimura *et al.* (2002)[17] (Japan, 72.20% in milk samples), El-Gohary *et al.* (2015)[18] (Egypt, 21.70% in milk samples), Saeidi and Sheikhshahrokh (2016)[19] (Iran, 21.90% in milk and 26.25% in meat samples). Ghorbani *et al.* (2016)[20] reported that the prevalence of *H. pylori* in food items were 20%. They showed that vegetable sandwich (45%), minced meat (32%) and meat sandwich (20%) were the most commonly contaminated samples.

Our results showed that ovine milk samples had the highest prevalence of *H. pylori*. This substance has been accepted by other researchers.[10,11,13,16,19] It is maybe due to the high ability of the sheep stomach to keep *H. pylori* and then its transmission into the environment. The main reason for the high prevalence of *H. pylori* in dairy samples is the fact that milk has an appropriate conditions and especially pH and activated water (AW) which support the growth and survival of *H. pylori* strains. Traditional cheese had also high prevalence of *H. pylori* and it is maybe due to the fact that the milk which used for preparation of cheese should not heat higher than 40°C. Therefore, *H. pylori* and also other types of bacteria can survive in this type of traditional dairy.

VacAs1a, *m1a*, *s2* and *m2* genotypes had a considerable prevalence in *H. pylori* strains. Similar findings have been reported previously in milk,[10,16,19] meat,[9,11,16] vegetable[9] and ready to eat foods.[20] Hemmatinezhad *et al.* (2016)[21] reported that the prevalence of *H. pylori* in various types of ready to eat food samples were 13.45%. They showed that olvie salad (36%), restaurant salad (30%), fruit salad (28%) and soup (22%) had the highest prevalence rate. Their findings reported that the most commonly detected combined genotypes were *s1am2* (70.27%), *s1am1a* (39.18%) and *m1am2* (31.08%) which was similar to our findings. Yahaghi *et al.* (2014)[9] revealed that *cagA* (57.62%), *vacA s1a* (37.28%) and *vacA m1a* (30.50 %) had the highest prevalence among the *H. pylori* strains of vegetables. High prevalence of *vacA* genotypes among the clinical isolates and cases of gastrointestinal disorders have been reported from Iran,[22] United States,[23] Australia,[24] United Kingdom[25] and China.[26] Adjacent connotation of *vacA* genotypes with production of interleukin 8 (IL-8) and cytotoxins, adhesion to gastric epithelial cells, occurrence of inflammation, vacuolization, necrosis and apoptosis of epithelial cells has been reported in previously published data.[27,28] High prevalence of these genotypes in milk and dairy samples of our investigation showed their high pathogenic nature.

Table 3. Distribution of *vacA* genotypes among the *Helicobacter pylori* strains of dairy products.

Samples (n. positive)	Distribution of *vacA* genotypes (%)						
	S1a	*S1b*	*S1c*	*S2*	*M1a*	*M1b*	*M2*
Bovine milk (13)	10 (76.92)	2 (15.38)	-	5 (38.46)	8 (61.53)	3 (23.07)	5 (38.46)
Ovine milk (18)	18 (100)	3 (16.66)	1 (5.55)	6 (33.33)	10 (55.55)	3 (16.66)	4 (22.22)
Traditional cheese (20)	19 (95)	5 (25)	2 (10)	7 (35)	13 (65)	4 (20)	6 (30)
Traditional cream (2)	2 (100)	-	-	1 (50)	2 (100)	-	1 (50)
Traditional butter (2)	2 (100)	-	-	1 (50)	1 (50)	-	1 (50)
Traditional ice-cream (5)	4 (80)	1 (20)	-	2 (40)	3 (60)	1 (20)	2 (40)
Total (60)	55 (91.66)	11 (18.33)	3 (5)	22 (36.66)	37 (61.66)	11 (18.33)	19 (31.66)

Table 4. Distribution of various combined alleles of the *vacA* genotypes of *Helicobacter pylori* isolated from dairy products.

Genotypes	Prevalence (%)*
S1am1a	25 (41.66)
S1am1b	7 (11.66)
S1am2	10 (16.66)
S1bm1a	4 (6.66)
S1bm1b	2 (3.33)
S1bm2	3 (5)
S1cm1a	1 (1.66)
S1cm1b	-
S1cm2	-
S2m1a	15 (25)
S2m1b	6 (10)
S2m2	8 (13.33)

*From a total of 60 *H. pylori* isolates.

Conclusions

Iranian raw milk and traditional dairy products harbor *H. pylori* strains with considerable distribution of *vacA* genotypes. Significant occurrence of *H. pylori* suggests that contaminated milk maybe the sources of *H. pylori* and their pathogenic genotypes. Similarity in the genotype pattern of *H. pylori* strains of various samples for the *vacA* alleles signifies their similar sources of infection. Simultaneous presence of these genotypes together in some of our strains showed their high pathogenicity. Regarding the high prevalence rate of pathogenic *H. pylori* beside the high consumption rate of milk and dairy products in Iran, serious public health hazard faced to the consumers.

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