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Prevalence and Genotyping of *Helicobacter pylori* Isolated From Meat, Milk and Vegetable in Iran

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Abstract

Background: Despite the considerable clinical role of *Helicobacter pylori*, its certain routes of transmission and origin have not been reported. Based on the argumentative hypothesis, foods play an imperative role in the spread of *H. pylori* to humans.
Objectives: The current research was done to investigate the prevalence rate and distribution of Vacuolating Cytotoxin A and Cytotoxin Associated Gene A genotypes in the *H. pylori* strains isolated from meat, milk, and vegetables.
Methods: A total of 340 food samples were collected and directly moved to the laboratory. Samples were cultured and *H. pylori* colonies were approved using the gram staining, urease test, and *16s rRNA*-based polymerase chain reaction (PCR) amplification. Positive strains were tested for distribution of *vacA* and *cagA* genotypes using the multiplex-PCR.
Results: Out of 340 samples, 40 (11.76%) harbored *H. pylori*. Prevalence of *H. pylori* in meat, milk, and vegetable samples were 7.33%, 16%, and 12.50%, respectively. Ovine milk (26%) was the most commonly contaminated sample. The most commonly detected genotypes were *vacA s1a* (87.50%), *vacA m1a* (87.50%), *vacA s2* (82.50%), *cagA* (80%), and *vacA m2* (62.50%). Genotypes of *S1am1a* (62.50%), *s2m1a* (55%), *s1am2* (50%), *s2m2* (45%), and *m1am2* (42.50%) were the most commonly detected combined genotypes.
Conclusions: Milk, vegetables, and meat, 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 for finding the exact sources of *H. pylori* strains.

Keywords: Prevalence, Genotypes, Milk, Meat, Vegetable, *Helicobacter pylori*

1. Background

Vegetable, meat, and milk are the most commonly consumed foods all around the world. High nutritional values of these foods for vitamins, mineral, fat, carbohydrate, protein, and other types of nutritional factors make them suitable for nutrition of children, youth, middle-aged, and elderly (1). Vegetables are in close contact with polluted soil, which is a source of many pathogenic agents. They are also irrigated with polluted non-drinking water and are mainly strengthened with human- and animal-based manures. Animal meat is mainly processed in a contaminated environment of slaughterhouses and are in close contact with blood, contents of the gastrointestinal tract, wool of animal, and also infected hands of butchers and meat inspectors. Animal milk is mainly achieved by traditional milking using hands and kept as well as transported in contaminated dishes. These options increase the possibility of their contamination with many types of pathogenic agents (2-4).

Helicobacter pylori is a microaerophilic and gram-negative bacterium, which is known as the causative agent of gastritis, peptic ulcer, adenocarcinoma, and lymphoma especially in the gastrointestinal tract (5). Documented data revealed that 20% to 90% of hospitalized patients with gastrointestinal disorders were infected with *H. pylori* strains (5, 6). In keeping with this, there were no previously recorded data regarding the main sources and also route of transmission of *H. pylori* into human (7). Several previously published data revealed that *H. pylori* had a significant prevalence in various types of foods, especially milk, meat, salad, vegetables, and ready to eat foods, which may show the food-borne route of this bacterium (8-10).

To evaluate the epidemiology of *H. pylori*, assessment of genotypes is important. Vacuolating Cytotoxin A (*vacA*) and Cytotoxin Associated Gene A (*cagA*) are the most important virulence markers detected in the *H. pylori* strains isolated from the cases of gastrointestinal disorders and also foods (11). Vacuolating Cytotoxin A gene is polymorphic and has variable structures. This gene is including mutable signal regions (*s1* and *s2*) and mid-regions (*m1* and *m2*). The *s1* region is additionally subtyped into *s1a*, *s1b*, as well as *s1c*, and the *m1* into *m1a* and *m1b* subtypes. Cytotoxin associated gene A is mainly associated with occurrence of

ulceration, inflammation and carcinoma (11, 12). It has also been detected in the samples taken from foods and also clinical gastrointestinal disorders (11, 12).

2. Objectives

According to the uncertain role of foods in the transmission of *H. pylori* to humans, as well as based on the lack of epidemiological and microbiological researches in this field in Iran, the present study was done in order to assess the prevalence rate and genotyping of *vacA* and *cagA* alleles of *H. pylori* strains isolated from raw meat, milk, and vegetable samples in Iran.

3. Methods

3.1. Ethics Statement

The study was approved by the ethical committee of research of the faculty of veterinary Medicine of the Islamic Azad University of Karaj, Iran (Consent Ref Number 95 - 210). Verification of this research project and the licenses related to the sampling process were approved by Dr. Zohreh Mashak (Approval Ref Number FST-95-210).

3.2. Sample Collection

From May 2016 to December 2016, a total of 340 food samples, including bovine raw meat (n = 50), ovine raw meat (n = 50), caprine raw meat (n = 50), bovine raw milk (n = 50), ovine raw milk (n = 50), caprine raw milk (n = 50), and raw vegetables (n = 40) were collected from the shopping centers of the Alborz province, Iran. Samples (200 g) were directly transported to the laboratory at 4 0C. All samples were kept under refrigeration in plastic bags.

3.3. Isolation of Helicobacter pylori

Twenty-five milligrams of each homogenized sample were added to 225 mL of Wilkins Chalgren anaerobe broth (Oxoid, UK) supplemented with 5% of horse serum (Sigma, St. Louis, MO, USA), colistin methanesulfonate (30 mg/L) (Oxoid, UK) and several types of antibiotic agents including nalidixic acid (Oxoid, UK), and trimethoprim (30 mg/L) (Oxoid, UK), cycloheximide (100 mg/L) (Oxoid, UK), and vancomycin (10 mg/L) (Oxoid, UK). All media were then incubated on microaerophilic conditions at 37°C for 7 days. Then, 0.1 mL of media was transmitted onto Wilkins Chalgren anaerobe agar (Oxoid, UK) supplemented with above mentioned materials. All media were then incubated on microaerophilic conditions at 37°C for 7 days. Colonies were then approved using the gram staining, urease test, as well as PCR based amplification of 16S *rRNA* specific gene of the *H. pylori* (HP-F: 5'-CTGGAGAGACTAAGCCCTCC-3' and HP-R: 5'-ATTACTGACGCTGATTGTGC-3) (110 bp) (Bioneer, South Korea) (13).

3.4. Genotyping of vacA and cagA Genotypes

DNA was extracted from the bacterial colonies using the DNA extraction and purification kit (Fermentas, Germany). Protocol was done according to the manufacture's instruction. Multiplex PCR was used to study the distribution of *vacA* (*s1a*, *s1b*, *s1c*, *s2*, *m1a*, *m1b* and *m2*) and *cagA* genotypes. List of primers and PCR condition is shown in Table 1 (14, 15). All runs were done using a programmable thermal cycler (Eppendorf-Netheler-Hinz GmbH, Germany) PCR device. Amplified PCR products (15 μL) were subjected to electrophoresis in 1.5% agarose gel in 1X TBE buffer at 80 V for 30 minutes, stained with CYBR Green (Fermentas, Germany). UVIdoc gel documentation system (UVIDoc, UK) was used for gel analysis. All runs comprised PCR grade water (Fermentas, Germany) as a negative control and also positive control (26695, J99, SS1, Tx30, 88-23 and 84-183) (Razi, Iran).

3.5. Statistical Analysis

Microsoft Excel software (Microsoft Corp., Redmond, WA, USA) was used for data analysis. Statistical analysis was done using the SPSS 21.0 statistical software (SPSS Inc., Chicago, IL, USA). Chi-square test and Fisher's exact 2-tailed tests were used to assess any significant relationship between prevalence of *H. pylori* strains and their genotypes. P value < 0.05 was considered as statistical significant level.

4. Results

Table 2 represents the total distribution of *H. pylori* in various types of food samples. We found that the total prevalence of *H. pylori* in various types of food samples was 11.76%. Prevalence of *H. pylori* in raw meat, milk, and vegetable samples were 7.33%, 16%, and 12.50%, respectively. Ovine milk had the highest prevalence of *H. pylori* (26%), while bovine meat had the lowest (4%). Statistically significant difference was seen between the types of samples and prevalence of *H. pylori* ($P < 0.05$).

Table 3 represents the distribution of various genotypes in the *H. pylori* strains of various types of food samples. We found that *vacA s1a* (87.50%), *vacA m1a* (87.50%), *vacA s2* (82.50%), *cagA* (80%), and *vacA m2* (62.50%) were the most commonly detected genotypes in *H. pylori* strains. Ovine samples had the most variables and also the highest prevalence of all studied genotypes. A statistically significant difference was seen between the source of samples and distribution of genotypes ($P < 0.05$).

Table 4 represents the total distribution of combined genotypes in the *H. pylori* isolates of food samples. We found that *s1am1a* (62.50%), *s2m1a* (55%), *s1am2* (50%), *s2m2* (45%), and *m1am2* (42.50%) were the most commonly detected combined genotypes. Genotypes of *s1cm1a* (2.50%),

Table 1. Oligonucleotide Primers, Volume, and Programs of PCR Reactions is Used for Genotyping of *vacA* and *cagA* Alleles of *Helicobacter pylori* Strains of Milk, Meat, and Vegetable Samples (14, 15)

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, 1.5 mM $Mgcl_2$, 200 μM dNTP (Fermentas), 0.5 μM of each primers F and R, 1.25 U Taq DNA polymerase (Fermentas), 2.5 μL DNA template	1 cycle: 95°C - 1 min. 32 cycle: 95°C - 45 s,64°C - 50 s, 72°C - 70 s. 1 cycle: 72°C - 5 min.
	R: CTGCTTGAATGCGCCAAAC			
vacA s1b	F: AGCGCCATACCGCAAGAG	187		
	CTGCTTGAATGCGCCAAAC			
vacA s1c	F: CTCTCGCTTTAGTGGGGYT	213		
	R: CTGCTTGAATGCGCCAAAC			
vacA s2	F: GCTAACACGCCAAATGATCC	199		
	R: CTGCTTGAATGCGCCAAAC			
vacA m1a	F: GGTCAAAATGCGGTCATGG	290		
	R: CCATTGGTACCTGTAGAAAC			
vacA m1b	F: GGCCCCAATGCAGTCATGGA	291		
	R: GCTGTTAGTGCCTAAAGAAGCAT			
vacA m2	F: GGAGCCCCAGGAAACATTG	352		
	R: CATAACTAGCGCCTTGCA			
cagA	F: GATAACAGCCAAGCTTTTGAGG	300	5 μL PCR buffer 10X, 2 mM $Mgcl_2$, 150 μM dNTP (Fermentas), 0.75 μM of each primers F and R, 1.5 U Taq DNA polymerase (Fermentas), 3 μL DNA template	1 cycle: 94°C - 1 min. 32 cycle: 95°C - 60 s, 56°C - 60 s, 72°C - 60 s. 1 cycle: 72°C - 10 min.
	R: CTGCAAAAGATTGTTTGGCAGA			

Table 2. Total Distribution of *Helicobacter pylori* in Various Types of Food Samples

Types of Samples	No. Samples Collected	Positive Results for *H. pylori* (%)
Meat		
Bovine	50	2 (4)
Ovine	50	5 (10)
Caprine	50	4 (8)
Total	150	11 (7.33)
Milk		
Bovine	50	4 (8)
Ovine	50	13 (26)
Caprine	50	7 (14)
Total	150	24 (16)
Vegetable	40	5 (12.50)
Total	340	40 (11.76)

s1cm1b (2.50%), and *s1cm2* (2.50%) had the lowest distribution among the *H. pylori* isolates of food samples.

5. Discussion

Results of the present study showed that *H. pylori* had a considerable prevalence in milk, vegetable, and meat samples. Results also indicated the high distribution of putative genotypes in *H. pylori* isolates of milk, meat, and vegetables. Total prevalence of *H. pylori* was 11.76%. This levels of prevalence of *H. pylori* in food samples was higher than that of Rahimi and Kheirabadi (2012) (16) (Iran, 0.67% in milk samples), Gilani et al. (2017) (17) (Iran, 5% in meat samples) and Atapoor et al. (2014) (4) (9.56% in vegetable), while was lower than that of Talaei et al. (2015) (18) (Iran, 4.76% - 20% in milk samples), Safaei et al. (2011) (19) (Iran, 16% in milk samples), Fujimura et al. (2002) (20) (Japan, 72.20% in milk samples), Esmaeiligoudarzi et al. (2015) (21) (Iran, 13.75% in milk samples and dairy products), El-Gohary et al. (2015) (22) (Egypt, 21.70% in milk samples), Saeidi and Sheikhshahrokh (2016) (23) (Iran, 21.90% in milk and 26.25% in meat samples), Mousavi et al. (2014) (24) (Iran, 19.80% in milk samples and 19.20% in dairy products) and Yahaghi et al. (2014) (25) (Iran, 14% in salad and 13.68% in vegetable).

Ghorbani et al. (2016) (26) reported that the prevalence of *H. pylori* in food items were 20%. They showed that vegetable sandwiches (45%), minced meat (32%), and meat sandwiches (20%) were the most commonly contaminated samples. Our results showed that ovine-based samples had the highest prevalence of *H. pylori*. This matter has been approved by other researchers (16-18, 23, 24). It may be due to the high ability of sheep stomach to keep *H. pylori* and its transmission into the environment. We found that milk samples had a higher prevalence of *H. pylori* than meat and vegetables. The main reason for the high prevalence of *H. pylori* in milk samples is the fact that milk has appropriate conditions, especially pH and activated water (aw), which support the growth and survival of *H. pylori* strains. On the other hand, bad conditions of meat and vegetables and maybe presence of antimicrobial agents in these foods make them unsuitable for growth and survival of *H. pylori*

Table 3. Distribution of Various Genotypes in *Helicobacter Pylori* Strains of Food Samples

Types of Samples (No. Positive)	Distribution of Genotypes (%)							
	S1a	*S1b*	*S1c*	*S2*	*M1a*	*M1b*	*M2*	*CagA*
Meat								
Bovine (2)	1 (50)	-	-	1 (50)	1 (50)	-	1 (50)	1 (50)
Ovine (5)	5 (100)	2 (40)	1 (20)	5 (100)	5 (100)	2 (40)	3 (60)	4 (80)
Caprine (4)	3 (75)	1 (25)	-	3 (75)	3 (75)	1 (25)	2 (50)	3 (75)
Total (11)	9 (81.81)	3 (27.27)	1 (9.09)	9 (81.81)	9 (81.81)	3 (27.27)	6 (54.54)	8 (72.72)
Milk								
Bovine (4)	2 (50)	-	-	2 (50)	2 (50)	-	1 (25)	2 (50)
Ovine (13)	13 (100)	5 (38.46)	3 (23.07)	13 (100)	13 (100)	7 (53.84)	10 (76.92)	12 (92.30)
Caprine (7)	6 (85.71)	1 (14.28)	1 (14.28)	5 (71.42)	6 (85.71)	1 (14.28)	4 (57.14)	6 (85.71)
Total (24)	21 (87.50)	6 (25)	4 (16.66)	20 (83.33)	21 (87.50)	8 (33.33)	15 (62.50)	20 (83.33)
Vegetable (5)	5 (100)	3 (60)	1 (20)	4 (80)	5 (100)	1 (20)	4 (80)	4 (80)
Total (40)	35 (87.50)	12 (30)	6 (15)	33 (82.50)	35 (87.50)	12 (30)	25 (62.50)	32 (80)

Table 4. Distribution of Combined Genotypes of *Helicobacter Pylori* Isolated from Various Types of Food Samples

Genotypes	Prevalence (%)[a]
S1am1a	25 (62.50)
S1am1b	5 (12.50)
S1am2	20 (50)
S1bm1a	4 (10)
S1bm1b	2 (5)
S1bm2	4 (10)
S1cm1a	1 (2.50)
S1cm1b	1 (2.50)
S1cm2	1 (2.50)
S2m1a	22 (55)
S2m1b	5 (12.50)
S2m2	18 (45)
M1am2	17 (42.50)
M1bm2	4 (10)
CagA+	32 (80)
CagA-	8 (20)

[a] From a total of 40 positive strains of *H. pylori*.

strains. High prevalence of *H. pylori* in vegetable samples is their close contact with polluted soil, water, and human- and animal-based manure. In addition, vegetables are not washed sufficiently in the shopping center and therefore, polluted materials remain even after washing. In addition, vegetables had so many structural wrinkles, which are considered as a shelter for pathogenic bacteria like *H. pylori*.

Another part of our study focused on the genotyping of bacterial strains. We found that *vacAs1a*, *m1a*, *s2* and *m2*, as well as *cagA* genotypes had a considerable prevalence in *H. pylori* strains. Similar findings have been reported previously in milk (16, 23, 24), meat (17, 23, 25), vegetables (25) and ready to eat foods (26). Hemmatinezhad et al. (2016) (27) 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) (25) revealed that *cagA* (57.62 %), *vacA* s1a (37.28 %), and *vacAm1a* (30.50 %) had the highest prevalence among the *H. pylori* strains of vegetables. High prevalence of *vacA* and *cagA* genotypes among clinical isolates and cases of gastrointestinal disorders have been reported from Iran (28), United States (29), Australia (30), United Kingdom (31), and China (32). Adjacent connotation of *cagA* and *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 (33, 34). High prevalence of these genotypes in milk, vegetables, and meat samples of our investigation showed their high pathogenic nature.

6. Conclusions

Iranian milk, meat, and vegetable samples harbor *H. pylori* strains with considerable distribution of *vacA* and *cagA* genotypes. Considerable incidence of *H. pylori* proposes that contaminated milk, vegetable, and meat may be the sources of *H. pylori* and their pathogenic genotypes. Similarity in the genotyping pattern of *H. pylori* strains of various samples represents 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, meat, and vegetables among Iranian people represent an important public health issue, which should be addressed before the vast spread of the *H. pylori* infection.

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Footnotes

Authors' Contribution: Zohreh Mashak designed the study and were responsible for the overall study management. Zohreh Mashak and Ali Talimkhani carried out the sampling, bacterial isolation, and PCR genetic alignment. Zohreh Mashak prepared the manuscript. All the authors read and accepted the final manuscript.

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