Phenotypic analysis of antibiotic resistance and genotypic study of the vacA, cagA, iceA, oipA and babA genotypes of the Helicobacter pylori strains isolated from raw milk

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Abstract

Background: Foods with animal origins and particularly milk play a considerable role in transmission of Helicobacter pylori. The current study was performed to assess phenotypic characters of antibiotic resistance and genotyping pattern of vacA, cagA, iceA, oipA and babA2 alleles amongst the H. pylori strains isolated from raw milk.

Methods: Six-hundred and thirty raw milk samples were collected and cultured on Wilkins Chalgren anaerobe media. Antibiotic resistance and genotyping patterns were studied using disk diffusion and PCR, respectively.

Results: Sixty-seven out of 630 (10.63%) raw milk samples were positive for H. pylori. Ovine raw milk (17.27%) samples had the highest prevalence of H. pylori, while camel (5.00%) had the lowest. H. pylori strains harbored the highest prevalence of resistance against ampicillin (82.08%), tetracycline (76.11%), amoxicillin (74.62%), metronidazole (65.67%) and erythromycin (53.73%). Prevalence of resistance against more than 10 types of antibiotics was 17.91%. VacA s1a (83.58%), m1a (80.59%), s2 (77.61%) and m2 (68.65%), cagA (73.13%) and babA2 (44.77%) were the most commonly detected genotypes. We found that S1am1a (56.71%), s2m1a (56.71%), s1am2 (43.28%) and s2m2 (43.28%) were the most commonly detected genotyping pattern. Frequency of cagA-, oipA- and babA2- genotypes were 26.86%, 62.68% and 55.22%, respectively. We found that S1a/cagA+/iceA1/oipA−/babA2- (28.35%), m1a/cagA+/iceA1/oipA−/babA2- (28.35%) and s2/cagA+/iceA1/oipA−/babA2- (28.35%) were the most commonly detected combined genotyping pattern.

Conclusions: Simultaneous presence of vacA, cagA, iceA, oipA and babA2 genotypes in antibiotic resistant H. pylori strains indicates important public health issue regarding the consumption of raw milk. However, additional researches are required to find molecular genetic homology and other epidemiological aspects of H. pylori in milk.

Keywords: Helicobacter pylori, Raw milk, Antibiotic resistance, Genotyping

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Background

Milk of animals provide a package of key nutrients that are difficult to obtain in diets with limited or no dairy products [1]. Dissimilarly, raw milk is not necessarily safe, as evidenced by higher rates of foodborne illnesses associated with its consumption [2–6]. Likewise, there were so many investigations about the considerable prevalence of some specific foodborne pathogens in raw milk [2–6].

*Helicobacter pylori* (*H. pylori*) is a microaerophilic and Gram-negative spiral coccoid flagellated bacterium with 2 to 4 μm in length and 0.5 to 1 μm in width. It is known as one of the main causative agents of duodenal ulcer, peptic ulcer disease, gastric adenocarcinoma, type B gastritis and gastric B-cell lymphoma [7–9]. Human stomach is considered as a main reservoir of *H. pylori* strains [7–9]. In keeping with this, foods with animal origins may play an imperative role in transmission of *H. pylori* infections to human [7–9]. Suitable conditions including pH, activated water (AW), moisture and temperature cause *H. pylori* to easily survive in milk [10]. Raw milk [10], pasteurized milk [7–9] and even sterilized food samples [10] have been introduced as possible emerging sources of *H. pylori* infections. Vacuolating Cytotoxin A (*vacA*) and Cytotoxin Associated Gene A (*cagA*) are two important virulence genes with high importance in the pathogenicity of *H. pylori* infections [7–10]. The *vacA* gene is polymorphic, comprising variable signal regions (type *s1* or *s2*) and mid-regions (type *m1* or *m2*). The *s1* type is additionally divided into *s1a*, *s1b* and *s1c* and the *m1* into *m1a* and *m1b* subtypes. The *cagA* gene has been detected in the severe cases of gastrointestinal disorders and peptic ulcers [7–10]. Induced by contact with the epithelium antigen (*iceA*), outer inflammatory protein (*oip*) and blood group antigen-binding adhesin gene (*babA*) are other important pathogenic genotypes of the *H. pylori* strains [7–11]. Genotyping using these virulence markers is considered as one of the best approaches to study the correlations between *H. pylori* isolates from different samples [11].

Antibiotic therapy is one of the best aspects of treatments for *H. pylori* infections. However, therapeutic options have become somewhat restricted because of the presence of severe resistance in some strains of this bacterium [12]. Documented data disclosed that *H. pylori* strains harbored the high prevalence of resistance against different types of antibiotics [12].

Data on the epidemiology and transmission of *H. pylori* is extremely significant in order to prevent its distribution and to identify high-risk populations, especially in areas that have high rates of infections such as Iran [7–10, 13, 14]. Considering the indistinct epidemiological aspects of *H. pylori* in milk and due to the high prevalence of *H. pylori* all-around the world [7–14], the present investigation was performed in order to study the prevalence rate, genotyping patterns and phenotypic evaluation of antibiotic resistance of the *H. pylori* strains isolated from raw milk samples of bovine, ovine, caprine, buffalo and camel.

### Methods

#### Samples

From January to March 2018, total 630 raw milk samples of bovine (*n* = 120), ovine (*n* = 110), caprine (*n* = 130), buffalo (*n* = 130) and camel (*n* = 140) were arbitrarily collected from the supermarkets of diverse areas of Isfahan province, Iran. All milk samples were collected from traditional dairy farms. Milk samples were kept at refrigerator. Throughout milk collection, the first few squirts were overlooked. The animals which their milk samples collected for this research were clinically healthy, and the milk samples displayed natural physical (color, odor, pH, and density) constancy. Samples (50 ml, in sterile glass bottles) were transported in ice-cooled flasks (at 4 °C) to the laboratory within two hours after collection.

#### Isolation of helicobacter pylori

Isolation of *H. pylori* bacteria was performed using the culture technique [7–10, 13, 14]. Twenty-five milliliters of milk sample were used for this purpose. Wilkins Chalgren anaerobe broth (Oxoid Ltd., Basingstoke, UK) was used for this purpose. Microaerophilic conditions (5% oxygen, 85% nitrogen and 10% CO2) was prepared using the MART system (MART system, Lichtenvoorde, The Netherlands).

#### DNA extraction and 16S rRNA-based PCR confirmation

Distinctive colonies of *H. pylori* were additionally approved using the 16S rRNA-based PCR method. Typical colonies were sub-cultured on Wilkins Chalgren anaerobe broth supplemented with same materials mentioned above [15]. Genomic DNA was then extracted from colonies using a DNA extraction kit (Thermo Fisher Scientific, St. Leon-Rot, Germany). Procedure was performed rendering to the manufacturer’s guidelines. Purity (A260/A280) and concentration of extracted DNA were then checked (NanoDrop, Thermo Scientific, Waltham, MA, USA). The truth of the DNA was assessed on a 2% agarose gel stained with ethidium bromide (0.5 μg/mL) (Thermo Fisher Scientific, St. Leon-Rot, Germany). Polymerase Chain Reaction (PCR) was performed using a PCR thermal cycler (Eppendorf Co., Hamburg, Germany) according to reported procedure [15].

#### Study the antibiotic resistance pattern

There are no accepted standardized methods for testing *H. pylori* antimicrobial susceptibilities and the protocols
used in this study were based on recently published guidelines [16] and also those of Performance Standards for Antimicrobial Susceptibility Testing- Clinical and Laboratory Standards Institute - NCCLS, 2007 [17]. Briefly, bacterial suspensions were adjusted to the 0.5 McFarland standard (equivalent to 1–2 × 10⁸ cfu/ml) and were used to inoculate Muller Hinton agar plates (Merck, Germany). Antimicrobial disks (ampicillin (10 µg), levofloxacin (5 µg), metronidazole (5 µg), clarithromycin (2 µg), amoxicillin (10 µg), streptomycin (10 µg), cefsulodin (30 µg), erythromycin (5 µg), tetracycline (30 µg), trimethoprim (25 µg), furazolidone (1 µg), rifampin (30 µg), and spiramycin (100 µg) (Oxoid, UK)) were applied and the plates were incubated under microaerophilic conditions at 35 °C for 16–18 h. The zones of growth inhibition produced by each antibiotic were measured and interpreted by standard procedure. Reference strains NCTC 13206 (CCUG 38770) and NCTC 13207 (CCUG 38772) were included as quality controls [18].

Genotyping analysis
Frequency of vacA, cagA, iceA, oipA and babA alleles were assessed using PCR [19–22]. Table 1 characterizes the set of primers and PCR circumstances applied for genotyping of vacA, cagA, iceA, oipA and babA alleles. Initially, all samples were subjected to pre-tests to found suitable time, temperature and volume of reaction. A programmable DNA thermo-cycler (Eppendorf Master-cycler 5330, Eppendorf-Nethel-Hinz GmbH, Hamburg, Germany) was used in all PCR reactions. PCR grade water and H. pylori standard strains (SS1, 26,695, Tx30, J99, 88–23 and 84–183) were used as negative and positive controls, respectively. Ten microliters of PCR product were exposed to electrophoresis in a 2% agarose gel in 1X TBE buffer at 80 V for 30 min, stained with SYBR Green. The UV1 doc gel documentation systems (Grade GB004, Jencons PLC, London, UK) was applied for analysis of images.

Statistical analysis
Data were subjected to Microsoft office Excel (version 15; Microsoft Corp., Redmond, WA, USA). Statistical analysis was performed by means of the SPSS 21.0 statistical software (SPSS Inc., Chicago, IL, USA). Chi-square test and Fisher’s exact two-tailed test were applied to measure any significant relationship. P value < 0.05 was considered as statistical significant level.

Results
Table 2 represents the prevalence of H. pylori in different types of raw milk samples. Sixty-seven out of 630 (10.63%) raw milk samples were positive for H. pylori strains. All isolates were also approved by the 16SrRNA gene PCR amplification. Ovine (17.27%) and caprine (13.84%) raw milk samples had the highest prevalence of H. pylori strains, while camel (5.00%) had the lowest. Statistically significant difference was seen between type of samples and prevalence of H. pylori strains (P < 0.05).

Table 3 represents the antibiotic resistance pattern of H. pylori strains isolated from different types of raw milk samples. H. pylori strains harbored the highest prevalence of resistance against ampicillin (82.08%), tetracycline (76.11%), amoxicillin (74.62%), metronidazole (65.67%) and erythromycin (53.73%) antibiotic agents. Furthermore, H. pylori strains harbored the lowest prevalence of resistance against cefsulodin (13.43%), furazolidone (13.43%), spiramycin (16.41%) and streptomycin (23.88%). Moreover, prevalence of resistance against clarithromycin, levofloxacin, rifampin and trimethoprim antibiotic agents were 47.76%, 38.80%, 32.83% and 34.32%, respectively. Statistically significant difference was seen between type of samples and prevalence of antibiotic resistance (P < 0.05). Figure 1 represents the distribution of multi-drug resistant H. pylori strains isolated from different types of raw milk samples. We found that all of the H. pylori strains isolated from raw milk samples at least had resistance against 3 different types of antibiotics, while prevalence of resistance against more than 3 types of antibiotics (etc) was 94.02%.

Table 4 represents the distribution of genotypes amongst the H. pylori strains isolated from different types of raw milk samples. VacA s1a (83.58%), m1a (80.59%), s2 (77.61%) and m2 (68.65%), cagA (73.13%) and babA2 (44.77%) were the most commonly detected genotypes amongst the H. pylori strains isolated from different types of raw milk samples. VacA s1c (10.44%), m1b (28.35%) and s1b (32.83%), iceA2 (19.40%) and oipA (37.31%) had the lowest prevalence amongst the H. pylori strains isolated from different types of raw milk samples. Statistically significant difference was seen between type of samples and prevalence of genotypes (P < 0.05). Additionally, statistically significant difference was seen between the prevalence of iceA1 and iceA2 genotypes (P < 0.05).

Table 5 represents the genotyping pattern of H. pylori strains isolated from different types of raw milk samples. S1am1a (56.71%), s2m1a (56.71%), s1am2 (43.28%) and s2 m2 (43.28%) were the most commonly detected genotyping pattern of the vacA alleles of H. pylori strains isolated from different types of raw milk samples. Distribution of cagA-, oipA- and babA2- genotypes were 26.86%, 62.68% and 55.22%, respectively. We found that 10.44% of H. pylori strains harbored iceA1/iceA2 genotyping pattern. S1cm1b (1.49%), s1 cn2 (4.47%), s1bm1b (7.46%), s1cm1a (7.46%), s1bm2 (11.94%), s2m1b (16.41%) and s1bm1a (16.41%) had the lowest prevalence
amongst different genotyping patterns of \(H.\, pylori\) strains.

Table 6 represents the combined genotyping pattern of \(H.\, pylori\) strains isolated from different types of raw milk samples. There were no detected combined genotyping pattern of \(H.\, pylori\) strains isolated from different types of raw milk samples. There were no detected combined genotyping pattern of \(H.\, pylori\) strains isolated from different types of raw milk samples.

Table 1 | Set of primers and PCR circumstances applied for genotyping of \(vacA\), \(cagA\), \(iceA\), \(oipA\) and \(babA\) alleles

| Genes   | Primer Sequence (5’-3’) | Size of product (bp) | Volume of PCR reaction (50 μl) | PCR programs |
|---------|-------------------------|----------------------|---------------------------------|--------------|
| \(VacA\) \(s\) \(a\) | F: CTCCTGCTTTATGAGGAGC  
R: CTGCTTGATGCACGCAAC | 213 | 5 μL PCR buffer 10 x 1.5 mM Mgcl₂  
150 μM dNTP (Thermo Fisher Scientific, St. Leon-Rot, Germany)  
0.75 μM of each primers F & R  
1.5 U Taq DNA polymerase (Thermo Fisher Scientific, St. Leon-Rot, Germany) | 1 cycle: 72 °C ------------ 10 min  
31 cycle: 95 °C ------------ 8 s  
56 °C ------------ 13 s  
95 °C ------------ 8 s  
1 cycle: 72 °C ------------ 10 min |
| \(VacA\) \(s\) \(b\) | F: AGCCGACATGCGCAAGAG  
R: CTGCTTGCAAGGCAAAAC | 187 | 0.5 μM of each primers F & R  
1.25 U Taq DNA polymerase | 32 cycle: 95 °C ------------ 45 s  
64 °C ------------ 50 s  
1 cycle: 72 °C ------------ 70 s |
| \(VacA\) \(s\) \(c\) | F: CTCCTGCTTTATGGGGYT  
R: CTGCTTGATGCGCAAAAC | 213 | (Thermo Fisher Scientific, St. Leon-Rot, Germany)  
1 cycle: 72 °C ------------ 5 min |
| \(VacA\) \(s\) \(d\) | F: GCTACACCGCAATAATGC  
R: CTGCTTGATGCACGCAAC | 199 | 2.5 μL DNA template |
| \(VacA\) \(m\) \(a\) | F: GTGCCTGATGCGCAAAAC  
R: CCATTTGACAGGTCAGA | 290 | |
| \(VacA\) \(m\) \(b\) | F: GCCCCGAGTAGAACGTA  
R: GCTTGTGATGCTGCAAAAC | 291 | |
| \(VacA\) \(m\) \(c\) | F: GGAGCCCCAGGAAACACCTG  
R: CATCAAGCGCTGCA | 352 | |
| \(Cag\) \(A\) | F: GATAACAGCAAGCTTGGAGG  
R: CTGCAAAAGATTTGTTGGCAGA | 300 | 5 μL PCR buffer 10X  
2 mM Mgcl₂  
150 μM dNTP (Thermo Fisher Scientific, St. Leon-Rot, Germany)  
0.75 μM of each primers F & R  
1.5 U Taq DNA polymerase (Thermo Fisher Scientific, St. Leon-Rot, Germany)  
3 μL DNA template | 1 cycle: 72 °C ------------ 5 min  
31 cycle: 95 °C ------------ 8 s  
56 °C ------------ 13 s  
95 °C ------------ 8 s  
1 cycle: 72 °C ------------ 10 min |
| \(IceA\) \(a\) | F: GTGTTTTTACAACCGAATGCTC  
R: CTAATGACCGTCTTGCA | 247 | 5 μL PCR buffer 10 x 2 mM Mgcl₂  
150 μM dNTP (Thermo Fisher Scientific, St. Leon-Rot, Germany)  
0.75 μM of each primers F & R  
1.5 U Taq DNA polymerase (Thermo Fisher Scientific, St. Leon-Rot, Germany)  
3 μL DNA template | 1 cycle: 72 °C ------------ 10 min  
31 cycle: 95 °C ------------ 8 s  
56 °C ------------ 13 s  
95 °C ------------ 8 s  
1 cycle: 72 °C ------------ 10 min |
| \(IceA\) \(b\) | F: GCTTTGTAATACACAAATTAT  
R: TTRCCTATTNTTTAGTATT | 229/334 | 5 μL PCR buffer 10 x 2 mM Mgcl₂  
150 μM dNTP (Thermo Fisher Scientific, St. Leon-Rot, Germany)  
0.75 μM of each primers F & R  
1.5 U Taq DNA polymerase (Thermo Fisher Scientific, St. Leon-Rot, Germany)  
3 μL DNA template | 1 cycle: 72 °C ------------ 10 min  
31 cycle: 95 °C ------------ 8 s  
56 °C ------------ 13 s  
95 °C ------------ 8 s  
1 cycle: 72 °C ------------ 10 min |
| \(OipA\) | F: GTTCCCCGATGCTATGATT | 401 | 5 μL PCR buffer 10 x 2 mM Mgcl₂  
150 μM dNTP (Thermo Fisher Scientific, St. Leon-Rot, Germany)  
0.75 μM of each primers F & R  
1.5 U Taq DNA polymerase (Thermo Fisher Scientific, St. Leon-Rot, Germany)  
3 μL DNA template | 1 cycle: 72 °C ------------ 10 min  
31 cycle: 95 °C ------------ 8 s  
56 °C ------------ 13 s  
95 °C ------------ 8 s  
1 cycle: 72 °C ------------ 10 min |
| \(BabA\) | F: CCAAGCGCAACAAAAACCGT  
R: GCTTGTGATAAXACCGGTCGT | 105–124 | 5 μL PCR buffer 10 x 2 mM Mgcl₂  
150 μM dNTP (Thermo Fisher Scientific, St. Leon-Rot, Germany)  
0.75 μM of each primers F & R  
1.5 U Taq DNA polymerase (Thermo Fisher Scientific, St. Leon-Rot, Germany)  
3 μL DNA template | 1 cycle: 72 °C ------------ 10 min  
31 cycle: 95 °C ------------ 8 s  
56 °C ------------ 13 s  
95 °C ------------ 8 s  
1 cycle: 72 °C ------------ 10 min |
Prevalence of \( H. pylori \) in different types of raw milk samples

| Raw milk samples | No samples collected | \( N \) (% of \( H. pylori \) positive samples) | \( H. pylori \) 16SrRNA PCR confirmation (%) |
|------------------|----------------------|-----------------------------------------------|-----------------------------------------------|
| Bovine           | 120                  | 9 (7.50)                                      | 9 (7.50)                                      |
| Ovine            | 110                  | 19 (17.27)                                    | 19 (17.27)                                    |
| Caprine          | 130                  | 18 (13.84)                                    | 18 (13.84)                                    |
| Buffalo          | 130                  | 14 (10.76)                                    | 14 (10.76)                                    |
| Camel            | 140                  | 7 (5.00)                                      | 7 (5.00)                                      |
| Total            | 630                  | 67 (10.63)                                    | 67 (10.63)                                    |

Discussion

\( H. pylori \) is a common bacterium with high microbiological and clinical importance and about 50% of the world’s population, depending on the geographic location considered, has been estimated to have been infected with this organism. Despite the high incidence of the infection, the reservoir for \( H. pylori \) and the routes of infection are still indeterminate and various routes of transmission have been recommended [23]. Moreover, epidemiological investigations suggest that transmission of \( H. pylori \) between individuals happens both via the oral–oral and fecal–oral routes [23]. In keeping with this, fecal–oral transmission has more significant implications than since \( H. pylori \) may occur in food and water supplies subsequent to fecal contamination [24]. Besides, the isolation of \( H. pylori \) in drinking water [13, 14], raw vegetables [7, 9], salads [7, 9], meat [25, 26], ready to eat foods [27, 28], sterilized foods [29, 30] and foods with animal origin such as milk [31–35], suggests that these foods may act as vehicles for transmission of \( H. pylori \) to human population.

The present study was performed to assess the prevalence rate, genotyping patterns and antibiotic resistance properties of \( H. pylori \) strains isolated from different types of raw milk samples. Totally, 10.63% of raw milk samples were positive for \( H. pylori \) strains. Prevalence of \( H. pylori \) strains in raw milk samples of bovine, ovine, caprine, buffalo and camel were 7.50, 17.27, 13.84, 10.76 and 5.00%, respectively. Several studies have been conducted in this field. Talaei et al. (2015) [36] reported that the total prevalence of \( H. pylori \) strains amongst the cow, sheep, goat and buffalo milk samples were 16.00, 13.79, 4.76, 13.33 and 20.00%, respectively. Quaglia et al. (2008) [35] determined that the prevalence of \( H. pylori \) strains in sheep, cow and goat milk samples were 33.00%, 50.00% and 25.60%, respectively. Mousavi et al. (2014) [10] described that the prevalence of \( H. pylori \) strains in bovine, ovine, caprine, buffalo and camel milk samples were 16.66, 35.00, 28.00, 15.00 and 13.30%, respectively.

Foods presenting intrinsic factors, including water activity higher than 0.97 and pH ranging from 4.9 to 6.0 such as raw milk, theoretically could provide conditions for survival of \( H. pylori \) [7–9]. Therefore, it is not surprising that the \( H. pylori \) strains has the high prevalence in raw milk samples of our investigation. High prevalence rate of \( H. pylori \) in milk samples of different parts of Sudan had a range of 7 to 38%. Similar results have been reported for the high prevalence of \( H. pylori \) in milk samples from Japan (72.20%) [32], Greece (20.00%) [34], Italy (1.80%) [39] and Iran (16.00%) [40].
### Table 3: Antibiotic resistance pattern of *H. pylori* strains isolated from different types of raw milk samples

| Type of raw milk samples (N of *H. pylori* strains) | N (%) isolates resistant to each antibiotic |
|---------------------------------------------------|-------------------------------------------|
|                                                   | AM10<sup>a</sup> | Met5 | ER5 | CLR2 | AMX 10 | Tet30 | Lev5 | S10 | RIF30 | Cef30 | TRP25 | FZL1 | Spi100 |
| Bovine (9)                                        | 8 (88.88)      | 6 (66.66) | 4 (44.44) | 4 (44.44) | 7 (77.77) | 7 (77.77) | 3 (33.33) | 2 (22.22) | 3 (33.33) | 2 (22.22) | 3 (33.33) | 2 (22.22) | 3 (33.33) |
| Ovine (19)                                        | 18 (94.73)     | 16 (84.21) | 14 (73.68) | 13 (68.42) | 17 (89.47) | 18 (94.73) | 12 (63.15) | 7 (36.84) | 10 (52.63) | 4 (21.05) | 10 (52.63) | 3 (15.78) | 3 (15.78) |
| Caprine (18)                                      | 14 (77.77)     | 12 (66.66) | 9 (50) | 8 (44.44) | 13 (72.22) | 13 (72.22) | 6 (33.33) | 4 (22.22) | 5 (27.77) | 2 (11.11) | 5 (27.77) | 3 (16.66) | 3 (16.66) |
| Buffalo (14)                                      | 11 (78.57)     | 8 (57.14) | 8 (57.14) | 6 (42.85) | 10 (71.42) | 10 (71.42) | 4 (28.57) | 2 (14.28) | 3 (21.42) | 1 (7.14) | 4 (28.57) | 1 (7.14) | 2 (14.28) |
| Camel (7)                                         | 4 (57.14)      | 2 (28.57) | 1 (7.14) | 1 (7.14) | 3 (42.85) | 3 (42.85) | 1 (7.14) | 1 (7.14) | 1 (7.14) | – | 1 (7.14) | – | – |
| Total (67)                                        | 55 (82.08)     | 44 (65.67) | 36 (53.73) | 32 (47.76) | 50 (74.62) | 51 (76.11) | 26 (38.80) | 16 (23.88) | 22 (32.83) | 9 (13.43) | 23 (34.32) | 9 (13.43) | 11 (16.41) |

<sup>a</sup>AM10: ampicillin (10 μg), Met5: metronidazole (5 μg), ER5: erythromycin (5 μg), CLR2: clarithromycin (2 μg), AMX10: amoxicillin (10 μg), Tet30: tetracycline (30 μg), Lev5: levofloxacin (5 μg), S10: streptomycin (10 μg), RIF30: rifampin (30 μg), Cef30: cefsulodin (30 μg), TRP25: trimethoprim (25 μg), FZL1: furazolidone (1 μg) and Spi100: spiramycin (100 μg)
bacteria of human clinical specimens displayed boost prevalence of resistance against aminoglycosides, tetracyclines, penicillins, macrolides and metronidazole [47] which was parallel to our results. We also found that vacA s1a, s2, m1a and m2, cagA, iceA1, oipA and babA2 genotypes, s1m1a, s2m1a, s1 am2, s2 m2, cagA-, oipA- and babA2- patterns and s1a/cagA+/iceA1/oipA−/babA2-, m1a/cagA+/iceA1/oipA−/babA2-, s2/cagA+/iceA1/oipA−/babA2-, s1a/cagA+/iceA1/oipA−/babA2+, m1a/cagA+/iceA1/oipA−/babA2+, s2/cagA+/iceA1/oipA−/babA2+, s1a/cagA+/iceA1/oipA+/babA2- and m2/cagA+/iceA1/oipA−/babA2+ combined genotyping patterns were the most commonly detected virulence characters of _H. pylori_ strains isolated from raw milk samples. High prevalence of vacA, cagA, iceA1, oipA and babA2 genotypes was also reported in the _H. pylori_ strains isolated from clinical samples of human and animal species [48–51]. Furthermore, high prevalence of these genotypes has been reported in the _H. pylori_ strains isolated from different types of food samples [8–10, 14, 36, 42–45, 52]. Adjacent association of vacA, cagA, iceA1, oipA and babA2 genotypes of _H. pylori_ bacteria with secretion of interleukin-8 and cytotoxin, adhesion to gastric epithelial cells, occurrence of inflammatory effect, vacuolization, apoptosis procedure in gastric epithelial cells, peptic ulceration, increase acute neutrophilic infiltration, interleukin-10 secretion and inflammation, has been presented previously [48–50]. Since _H. pylori_ isolates in our investigation harbored vacA, cagA, iceA1, oipA and babA2 genotypes, therefore consumption of raw milk contaminated with virulent strains of _H. pylori_ may aggravate duodenal ulceration, gastric mucosal atrophy and gastric cancer. Additionally, some of _H. pylori_ isolates were simultaneously positive for more than one detected genotypes which poses their higher pathogenicity. Similar genotyping patterns of _H. pylori_ strains

**Fig. 1** Distribution of multidrug resistant _H. pylori_ strains isolated from different types of raw milk. Multidrug resistant _H. pylori_ strains were determined as those who had at least simultaneous resistance against 3 or more than 3 types of antibiotics.
Table 4 Distribution of genotypes amongst the *H. pylori* strains isolated from different types of raw milk samples

| Type of raw milk samples (N of *H. pylori* strains) | N (%) isolates harbor each genotype |
|---------------------------------------------------|-----------------------------------|
|                                                   | VacA s1a s1b s1c s2 m1a m1b m2 CagA keA1 keA2 OipA BobA2 |
| Bovine (9)                                        | 7 (77.77) 3 (33.33) 1 (11.11) 6 (66.66) 7 (77.77) 3 (33.33) 6 (66.66) 6 (66.66) 4 (44.44) 2 (22.22) 3 (33.33) 4 (44.44) |
| Ovine (19)                                        | 17 (89.47) 8 (42.10) 2 (10.52) 17 (89.47) 17 (89.47) 7 (36.84) 15 (78.94) 16 (84.21) 10 (52.63) 5 (26.31) 9 (50) 10 (52.63) |
| Caprine (18)                                      | 15 (83.33) 6 (33.33) 2 (11.11) 13 (72.22) 14 (77.77) 5 (27.77) 12 (66.66) 13 (72.22) 8 (44.44) 3 (16.66) 7 (38.88) 8 (44.44) |
| Buffalo (14)                                      | 12 (85.71) 4 (28.57) 1 (7.14) 12 (85.71) 12 (85.71) 3 (21.42) 10 (71.42) 11 (78.57) 6 (42.85) 2 (14.28) 5 (35.71) 6 (42.85) |
| Camel (7)                                         | 5 (71.42) 1 (14.28) 1 (14.28) 4 (57.14) 4 (57.14) 1 (14.28) 3 (42.85) 3 (42.85) 3 (42.85) 1 (14.28) 1 (14.28) 2 (28.57) |
| Total (67)                                        | 56 (83.58) 22 (32.83) 7 (10.44) 52 (77.61) 54 (80.59) 19 (28.35) 46 (68.65) 49 (73.13) 31 (46.26) 13 (19.40) 25 (37.31) 30 (44.77) |
| Type of raw milk samples (N of H. pylori strains) | Genotyping pattern (%) | s1am1a | s1am1b | s1am2 | s1bm1a | s1bm1b | s1bm2 | s1cm1a | s1cm1b | s1cm2 |
|------------------------------------------------|------------------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| Bovine (9)                                      |                        | 5 (55.55) | 2 (22.22) | 4 (44.44) | 2 (22.22) | 1 (11.11) | 2 (22.22) | 1 (11.11) | –      | 1 (11.11) |
| Ovine (19)                                      |                        | 13 (68.42) | 4 (21.05) | 11 (57.89) | 4 (21.05) | 2 (10.52) | 3 (15.78) | 1 (5.26) | 1 (5.26) | 1 (5.26) |
| Caprine (18)                                    |                        | 11 (61.11) | 3 (16.66) | 10 (55.55) | 2 (11.11) | 1 (5.55) | 2 (11.11) | 1 (5.55) | –      | 1 (5.55) |
| Buffalo (14)                                    |                        | 7 (50)     | 1 (7.14)  | 3 (21.42) | 2 (14.28) | 1 (7.14) | 1 (7.14) | 1 (7.14) | –      | –      |
| Camel (7)                                       |                        | 2 (28.57)  | –       | 1 (14.28) | 1 (14.28) | –       | –       | 1 (14.28) | –      | –      |
| Total (67)                                      |                        | 38 (56.71) | 10 (14.92) | 29 (43.28) | 11 (16.41) | 5 (7.46) | 8 (11.94) | 5 (7.46) | 1 (1.49) | 3 (4.47) |
Table 5 Genotyping pattern of *H. pylori* strains isolated from different types of raw milk samples (Continued)

| Type of raw milk samples (N of *H. pylori* strains) | Genotyping pattern (%) | s2m1a | s2m1b | s2 m2 | CagA+ | CagA- | IceA1/IceA2 | OipA+ | OipA- | BabA2+ | BabA2- |
|--------------------------------------------------|------------------------|-------|-------|-------|-------|-------|-------------|-------|-------|--------|--------|
| Ovine (19)                                        |                        | 15 (78.94) | 5 (26.31) | 12 (63.15) | 16 (84.21) | 3 (15.78) | 3 (15.78) | 9 (47.36) | 10 (52.63) | 10 (52.63) | 9 (47.36) |
| Caprine (18)                                      |                        | 10 (55.55) | 3 (16.66) | 8 (44.44) | 13 (72.22) | 5 (27.77) | 2 (11.11) | 7 (38.88) | 11 (61.11) | 8 (44.44) | 10 (55.55) |
| Buffalo (14)                                      |                        | 7 (50) | 1 (7.14) | 5 (35.71) | 11 (78.57) | 3 (21.42) | 1 (7.14) | 5 (35.71) | 9 (64.28) | 6 (42.85) | 8 (57.14) |
| Camel (7)                                         |                        | 2 (28.57) | – | 1 (14.28) | 3 (42.85) | 4 (57.14) | – | 1 (14.28) | 6 (85.71) | 2 (28.57) | 5 (71.42) |
| Total (67)                                        |                        | 38 (56.71) | 11 (16.41) | 29 (43.28) | 49 (73.13) | 18 (26.86) | 7 (10.44) | 25 (37.31) | 42 (62.68) | 30 (44.77) | 37 (55.22) |
Table 6 Combined genotyping pattern of *H. pylori* strains isolated from different types of raw milk samples (Continued)

| Combined genotyping patterns | Distribution (%) |
|------------------------------|------------------|
| s1a/cagA+/iceA1/oipA+ | 5 (7.46) |
| s1a/cagA+/iceA1/oipA+ | 6 (8.95) |
| s1a/cagA+/iceA1/oipA+ | 7 (10.44) |
| s1a/cagA+/iceA1/oipA+ | 8 (11.94) |
| s1a/cagA+/iceA1/oipA+ | 10 (14.92) |
| s1b/cagA+/iceA1/oipA+ | 4 (5.97) |
| s1b/cagA+/iceA1/oipA+ | 5 (7.46) |
| s1b/cagA+/iceA1/oipA+ | 5 (7.46) |
| s1b/cagA+/iceA1/oipA+ | 9 (13.43) |
| s1b/cagA+/iceA1/oipA+ | 11 (16.41) |
| s1b/cagA+/iceA1/oipA+ | 12 (17.91) |
| s1b/cagA+/iceA1/oipA+ | 14 (20.89) |
| s1b/cagA+/iceA1/oipA+ | 2 (2.98) |
| s1b/cagA+/iceA1/oipA+ | 3 (4.47) |
| s1b/cagA+/iceA1/oipA+ | 4 (5.97) |
| s1b/cagA+/iceA1/oipA+ | 5 (7.46) |
| s1b/cagA+/iceA1/oipA+ | 1 (1.49) |
| s1b/cagA+/iceA1/oipA+ | 1 (1.49) |
| s1b/cagA+/iceA1/oipA+ | 1 (1.49) |
| s1b/cagA+/iceA1/oipA+ | 2 (2.98) |
| s1b/cagA+/iceA1/oipA+ | 2 (2.98) |
| s1b/cagA+/iceA1/oipA+ | 2 (2.98) |
| s1b/cagA+/iceA1/oipA+ | 2 (2.98) |
| s1b/cagA+/iceA1/oipA+ | 1 (1.49) |
| s1b/cagA+/iceA1/oipA+ | 2 (2.98) |
| s1b/cagA+/iceA1/oipA+ | 1 (1.49) |
| s1b/cagA+/iceA1/oipA+ | 2 (2.98) |
| s1b/cagA+/iceA1/oipA+ | 2 (2.98) |
| s1b/cagA+/iceA1/oipA+ | 5 (7.46) |
| s1b/cagA+/iceA1/oipA+ | 8 (11.94) |
| s1b/cagA+/iceA1/oipA+ | 10 (14.92) |
| s1b/cagA+/iceA1/oipA+ | 2 (2.98) |
| s1b/cagA+/iceA1/oipA+ | 5 (7.46) |
| s1b/cagA+/iceA1/oipA+ | 8 (11.94) |
| s1b/cagA+/iceA1/oipA+ | 10 (14.92) |
| s1b/cagA+/iceA1/oipA+ | 13 (19.40) |
| s1b/cagA+/iceA1/oipA+ | 2 (2.98) |
| s1b/cagA+/iceA1/oipA+ | 3 (4.47) |
for a week has been recommended as the treatment of and clarithromycin, and a proton pump inhibitor given
H. pylori strains.
any significant relationship between the presence of resistance [60, 61]. Therefore, it is important to found
mycins and metronidazole resistance [59], while others
vacA revealed that the less virulent
ation between clarithromycin resistance mutations and
zole resistance [57]. Other studies have found an associ-
cagA found that neither cagA nor vacA was associated with
resistance [60, 61]. Therefore, it is important to found
any significant relationship between the presence of virulence markers and antibiotic resistance amongst the
H. pylori strains.

Triple therapy, including two antibiotics, amoxicillin and clarithromycin, and a proton pump inhibitor given for a week has been recommended as the treatment of
choice at several consensus conferences [62]. However, this treatment may fail for several reasons, as reported elsewhere [63]. In fact, the main reason for failure was found to be H pylori resistance to one of the antibiotics used (that is, clarithromycin). Other treatments have also been proposed, including metronidazole, a drug for which resistance is also a problem although to a lesser extent, as well as tetracycline, fluoroquinolones, and rifamycins for which resistance has become an emerging issue [64]. Results of the present investigation showed that application of furazolidone, streptomycin and cefsulodin may be effective for treatment of the cases of H. pylori infections. Reduction in the antibiotic prescription and also prescription of antibiotics according to the results of the disk diffusion can reduce the risk of antibiotic resistance. Using medicinal plants and especially those with high antimicrobial effects is a practical alternative way for treatment of H. pylori infection.

Conclusions
To put it in a nutshell, we recognized a great numbers of virulent and resistant H. pylori bacteria in raw milk samples of bovine, ovine, caprine, buffalo and camel species. Boost incidence of H. pylori bacteria in raw milk characterizes that these samples may be the natural reservoirs of the bacteria and can spread H. pylori to human. Moreover, some of the H. pylori bacteria of our research harbored vacA, cagA, iceA, oipA and babA2 genotypes together which represents the high pathogenicity. Furthermore, higher prevalence of iceA1+ strains than iceA2+, oipA+ and finally babA2+ is another important finding of our study. Additionally, presence of 97 diverse combined genotyping patterns with high distribution of s1a/cagA+/iceA1/oipA−/babA2+, m1a/cagA+/iceA1/oipA−/babA2−, s2/cagA+/iceA1/oipA−/babA2−, 44/cagA+/iceA1/oipA−/babA2−, s1a/cagA+/iceA1/oipA−/babA2−, m1a/cagA+/iceA1/oipA−/babA2−, s2/cagA+/iceA1/oipA−/babA2−, s1a/cagA+/iceA1/oipA−/babA2−, m2/cagA+/iceA1/oipA−/babA2−, s2/cagA+/iceA1/oipA−/babA2− is another interesting finding of our research. Similarities in the genotyping pattern of H. pylori strains between various milk sources represent their same route of infection. High prevalence of multi-drug resistant H. pylori strains shows that raw milk of bovine, ovine, caprine, buffalo and camel species may be reservoir of antibiotic resistant H. pylori. Prescription of cefsulodin, furazolidone, spiramycin and streptomycin may be effectual for treatment of cases of H. pylori infections due to the consumption of raw milk. Additional researches are essential to recognize the rates of the molecular genetic homology of H. pylori bacteria isolated from milk and dairy samples and those of human clinical specimens to confirm the zoonotic aspects of H. pylori.

Table 6 Combined genotyping pattern of H. pylori strains isolated from different types of raw milk samples (Continued)

| Combined genotyping patterns | Distribution (%) |
|-----------------------------|------------------|
| m1b/cagA+/iceA2/oipA−/babA2+ | 4 (5.97)         |
| m1b/cagA+/iceA2/oipA−/babA2− | 4 (5.97)         |
| m1b/cagA−/iceA1/oipA+/babA2+ | –                |
| m1b/cagA−/iceA1/oipA+/babA2− | 1 (1.49)         |
| m1b/cagA−/iceA1/oipA−/babA2+ | 1 (1.49)         |
| m1b/cagA−/iceA1/oipA−/babA2− | 2 (2.98)         |
| s1b/cagA−/iceA2/oipA+/babA2+ | –                |
| m1b/cagA−/iceA2/oipA+/babA2− | 1 (1.49)         |
| m1b/cagA−/iceA2/oipA−/babA2+ | 2 (2.98)         |
| m1b/cagA−/iceA2/oipA−/babA2− | 2 (2.98)         |
| m2/cagA+/iceA1/oipA+/babA2+ | 11 (16.41)       |
| m2/cagA+/iceA1/oipA+/babA2− | 13 (19.40)       |
| m2/cagA+/iceA1/oipA−/babA2+ | 15 (22.38)       |
| m2/cagA+/iceA1/oipA−/babA2− | 18 (26.86)       |
| m2/cagA+/iceA2/oipA+/babA2+ | 2 (2.98)         |
| m2/cagA+/iceA2/oipA+/babA2− | 4 (5.97)         |
| m2/cagA+/iceA2/oipA−/babA2+ | 5 (7.46)         |
| m2/cagA+/iceA2/oipA−/babA2− | 6 (8.95)         |
| m2/cagA−/iceA1/oipA+/babA2+ | 3 (4.47)         |
| m2/cagA−/iceA1/oipA+/babA2− | 6 (8.95)         |
| m2/cagA−/iceA1/oipA−/babA2+ | 8 (11.94)        |
| m2/cagA−/iceA1/oipA−/babA2− | 10 (14.92)       |
| m2/cagA−/iceA2/oipA+/babA2+ | 1 (1.49)         |
| m2/cagA−/iceA2/oipA+/babA2− | 3 (4.47)         |
| m2/cagA−/iceA2/oipA−/babA2+ | 3 (4.47)         |
| m2/cagA−/iceA2/oipA−/babA2− | 5 (7.46)         |

*Distribution was achieved based on the total numbers of 67 H. pylori isolates recovered from human clinical samples were also reported previously [53–66].

A possible relationship between virulence factors and antimicrobial resistance has been suggested. A study conducted in 2009 in Ireland reported that the absence of cagA may be a risk factor for developing metronidazole resistance [57]. Other studies have found an association between clarithromycin resistance mutations and the less virulent vacA genotypes [58]. Another report revealed that cagE and vacA S1 correlated with clarithromycin and metronidazole resistance [59], while others found that neither cagA nor vacA was associated with resistance [60, 61]. Therefore, it is important to found any significant relationship between the presence of virulence markers and antibiotic resistance amongst the H. pylori strains.
Abbreviations
BabA: Blood group Antigen-Binding Adhesin gene; CagA: Cytotoxin Associated Gene AlcEInduced by Contact with the Epithelium Antigen; H. pylori: Helicobacter pylori; Oip: Outer Inflammatory Protein; PCR: Polymerase Chain Reaction; SPSS: Statistical Package for the Social Sciences; VacA: Vacuolating Cytotoxin A

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Availability of data and materials
All data generated or analyzed throughout this research are included in this published article.

Authors’ contributions
FSD designed the study and carried out the PCR genetic alignment. RR supported the study and carried out the culture-based identification, disk diffusion and statistical analysis. FYF carried out the sample collection and preparation and writing of the manuscript. RR carried out the drafting of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
The study was approved by the Ethical Council of Research of the Molecular Biology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran (Consent Ref Number 97–23). Verification of this research project and the licenses related to sampling process were approved by the Prof. Reza Ranjbar (Approval Ref Number FHQC 2017/23).

Consent for publication
There was no consent for publication.

Competing interests
The authors declare that they have no competing interests.

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