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Molecular genotyping and antimicrobial resistance characters of *Helicobacter pylori* isolates from raw milk of naturally infected animal species

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ABSTRACT:
Raw milk of livestock species is considered a possible source of *H. pylori* transmission into humankind. The existing research was accomplished to measure the genotyping profile of *H. pylori* isolates of raw milk samples of naturally infected animal species. One-thousand and two-hundred raw milk samples were collected from livestock species. The microbial culture method has assessed the presence of *H. pylori* strains. The polymerase chain reaction established bacterial isolates. The genotyping profile was studied using the PCR. Two hundred and ten out of 1200 (17.50%) raw milk samples were contaminated with *H. pylori*. Raw ovine milk (24.21%) harbored the highest contamination rate. The most frequently identified genotypes were *vacA s1a* (64.76%), *s2* (57.14%), *m1a* (56.19%), and *m2* (54.28%), *cagA* (49.52%) and *cagE* (49.52%). The role of raw milk, predominantly ovine milk, was determined to transmit resistant and virulent *H. pylori* strains. *VacA*, *cagE*, and *cagA* genotypes were the most commonly detected. Higher distribution of *iceA1* than *iceA2*, *oipA*- than *oipA*+, *cagA*- than *cagA*+, and *cagE*- than *cagE*+ was another novel finding. *CagA*-, *CagE*-, and *oipA*- *H. pylori* had the higher frequency.

Keywords: Helicobacter pylori, Genotyping, Raw milk.

INTRODUCTION:

Milk is a popular and nutritious foodstuff among people all around the world. Its consumption in both raw and pasteurized forms is common among people (Lucey 2015). However, some people prefer to use only raw milk in their mills (Claeys et al. 2013). Recent researches showed that raw milk is not safe microbiologically, as established by advanced rates of food-borne diseases after its consumption (Dekordi et al. 2013; Rahimi et al. 2014; Dehkordi et al. 2014; Costard et al. 2017; Rahi et al. 2020; Keba et al. 2020).

Helicobacter pylori (*H. pylori*) is a contributing agent of gastric adenocarcinoma, peptic ulcer disease, duodenal ulcer, gastritis, and B-cell lymphoma (Kakiuchi et al. 2021). Although the human stomach is determined as the chief *H. pylori* source (Kakiuchi et al. 2021), it was routinely isolated from varied foods, chiefly those with animal origin (Zamani et al. 2017; Mezmale et al. 2020). Indeed, the livestock specie's raw milk is considered the most critical *H. pylori* reservoir (Talaei et al. 2015; Fusco et al. 2020).

H. pylori pathogenicity has been implicated with the presence of the number of genotypic factors encoding virulence. Among them, outer inflammatory protein (*oip*), Cytotoxin Associated Gene A and E (*cagA* and *cagE*), Vacuolating Cytotoxin A (*vacA*), and Induced by contact with the epithelium antigen (*iceA*) have been more detected in the *H. pylori* human clinical infections (Gilani et al. 2017a; Asl et al. 2020). The *vacA* gene consists of two separate mid- (*m1* and *m2*) and signals- (*s1* and *s2*) regions. The *s1* region is divided into a, b, and c, and the *m1* into a and b subtypes (Gilani et al. 2017a; Asl et al. 2020).

Several antimicrobial choices are available for *H. pylori* treatment (Saleem et al. 2020). However, *H. pylori* isolates of infections and foodstuffs harbored severe resistance toward commonly used antimicrobial agents in healthcare units (Sholeh et al. 2020). Thus, studying

the profile and pattern of antibiotic resistance amongst *H. pylori* isolates of foodstuffs as novel reservoirs of bacterial transmission to the human population seems essential.

Given the boost *H. pylori* incidence amongst Iranians (Keikha et al. 2020), the high importance of the bacteria as a virulence and antimicrobial-resistant agent, and finally, the high raw milk consumption in some parts of the world, an existing investigation was accomplished to measure the genotyping of *H. pylori* isolates of raw caprine, ovine, and bovine milk samples.

MATERILAS AND METHODS:

Samples:

An existing cross-sectional survey was performed in 2020 (April to October). One thousand and two hundred raw milk (ovine (n= 380), bovine (n= 420), and caprine (n=400)) samples were arbitrarily collected from the superstores of dissimilar areas of Iran. Collected raw milk samples harbored normal quality, particularly pH, odor, color, and density. Samples (80 mL, in a sterilized plastic bag) were transmitted at refrigerator temperature to the laboratory.

H. pylori identification:

Method labeled beforehand was applied for this goal (Rahimi and Kheirabadi 2012). Isolation was done on 25 mL of samples. Raw milk samples were cultured onto Wilkins Chalgren anaerobe broth (Oxoid, UK). To specify the criteria of *H. pylori* growth, cultures were supplemented with horse serum (5%) and several antimicrobials (cycloheximide (100 mg/L), nalidixic acid (30 mg/L), trimethoprim (30 mg/L), and vancomycin (10 mg/L)) (Sigma, USA). The MART system (Lichtenvoorde, The Netherland) was applied to prepare microaerophilic circumstances (nitrogen (85%), oxygen (5%), and CO_2 (10%)). Media were incubated at 37°C for 7 days. *H. pylori* (ATCC 43504) was applied as a control. *H. pylori* identification was performed by colony morphology and biochemical examinations, including Gram-staining, oxidase, urease, and catalase tests. *H. pylori* final confirmation was done by the Polymerase Chain Reaction (PCR) (Ho et al. 1991). DNA was isolated from colonies cultured in the same circumstances as mentioned above using the commercial kit (Thermo Fisher Scientific, St. Leon-Rot, Germany). The Nanodrop device was applied to assess the purity of extracted DNA (NanoDrop, Thermo Scientific, Waltham, MA, USA). Additionally, DNA quality was measured through electrophoresis. A thermal cycler (Eppendorf Co., Hamburg, Germany) was used in all runs. Positive (*H. pylori* (ATCC 26695)) and negative (PCR grade water, Thermo Fisher Scientific, Germany) controls were also applied.

Study the genotyping pattern:

Table 1 exhibits the PCR circumstances applied for the determination of *H. pylori iceA*, *vacA*, *oipA*, *cagA*, and *cagE* genotypes (Peek et al. 1998; Yamazaki et al. 2005; Wang et al. 2002). Controls (positives and negatives) were applied according to Rahi et al. (2020) (Rahi et al. 2020). The final PCR product (10 µL) was run by electrophoresis (2% agarose gel stained with SYBR Green for about 30 min at 80 V). The UVI doc device (Grade GB004, Jencons PLC, London, UK) was applied for final gel analysis.

Table 1. PCR circumstances applied to detect genotypes (Peek et al. 1998; Yamazaki et al. 2005; Wang et al. 2002).

Genes	Primer Sequence (5'-3')	Size of product (bp)	The volume of PCR reaction (50 µl)	PCR programs
VacA s_1a	F: CTCTCGCTTTAGTAGGAGC R: CTGCTTGAATGCGCCAAAC	213	10X PCR buffer: 5 µL $Mgcl_2$: 1.5 mM dNTP: 200 µM Primer F: 0.5 µM Primer R: 0.5 µM Taq DNA polymerase: 1.25 U DNA: 2.5 µL	1 cycle: 1 min: 95 ^{0C} 32 cycles: 45 s: 95 ^{0C} 50 s: 64 ^{0C} 70 s: 72 ^{0C} 1 cycle: 5 min: 72 ^{0C}
VacA s_1b	F: AGCGCCATACCGCAAGAG CTGCTTGAATGCGCCAAAC	187		
VacA s_1c	F: CTCTCGCTTTAGTGGGGYT R: CTGCTTGAATGCGCCAAAC	213		
VacA s_2	F: GCTAACACGCCAAATGATCC R: CTGCTTGAATGCGCCAAAC	199		
VacA m_1a	F: GGTCAAAATGCGGTCATGG R: CCATTGGTACCTGTAGAAAC	290		

VacA m_1b		F: GGCCCCAATGCAGTCATGGA R: GCTGTTAGTGCCTAAAGAAGCAT	291		
VacA m_2		F: GGAGCCCCAGGAAACATTG R: CATAACTAGCGCCTTGCA	352		
Cag A		F: GATAACAGCCAAGCTTTTGAGG R: CTGCAAAAGATTGTTTGGCAGA	300	10X PCR buffer: 5 µL $Mgcl_{2:}$ 1.5 mM dNTP: 200 µM Primer F: 0.5 µM Primer R: 0.5 µM Taq DNA polymerase: 1.25 U DNA: 2.5 µL	1 cycle: 1 min: 94 ^{0C} 32 cycles: 60 s: 95 ^{0C} 60 s: 56 ^{0C} 60 s: 72 ^{0C} 1 cycle: 10 min: 72 ^{0C}
IceA	*IceA1*	F: GTGTTTTTAACCAAAGTATC R: CTATAGCCASTYTCTTTGCA	247	10X PCR buffer: 5 µL $Mgcl_{2:}$ 1.5 mM dNTP: 200 µM Primer F: 0.5 µM Primer R: 0.5 µM Taq DNA polymerase: 1.25 U DNA: 2.5 µL	1 cycle: 1 min: 94 ^{0C} 32 cycles: 60 s: 94 ^{0C} 60 s: 56 ^{0C} 60 s: 72 ^{0C} 1 cycle: 10 min: 72 ^{0C}
	IceA2	F: GTTGGGTATATCACAATTTAT R: TTRCCCTATTTTCTAGTAGGT	229/334		
OipA		F: GTTTTTGATGCATGGGATTT R: GTGCATCTCTTATGGCTTT	401	10X PCR buffer: 5 µL $Mgcl_{2:}$ 1.5 mM dNTP: 200 µM Primer F: 0.5 µM Primer R: 0.5 µM Taq DNA polymerase: 1.25 U DNA: 2.5 µL	1 cycle: 1 min: 94 ^{0C} 32 cycles: 60 s: 94 ^{0C} 60 s: 56 ^{0C} 60 s: 72 ^{0C} 1 cycle: 10 min: 72 ^{0C}
cagE		F: TTGAAAACTTCAAGGATAGGATAGAGC R: GCCTAGCGTAATATCACCATTACCC	500	10X PCR buffer: 5 µL $Mgcl_{2:}$ 1.5 mM dNTP: 200 µM Primer F: 0.5 µM Primer R: 0.5 µM Taq DNA polymerase: 1.25 U DNA: 2.5 µL	1 cycle: 4 min: 95 ^{0C} 31 cycle: 44 s: 95 ^{0C} 45 s: 51 ^{0C} 62 s: 72 ^{0C} 1 cycle: 5 min: 72 ^{0C}

Data Analysis:
Data analysis was performed employing the SPSS 21.0 (Chicago, USA). Significant differences were studied according to the results of the Chi-square and Fisher's tests. P-value <0.05 was measured as a significant level.

RESULTS:

H. pylori incidence:

Table 2 expresses the H. pylori incidence amongst the examined samples. Two-hundred and ten out of 1200 (17.50%) examined samples were contaminated with H. pylori. Raw ovine milk (24.21%) harbored the maximum H. pylori contamination rate, while bovine raw milk (11.90%) harbored the lowermost. H. pylori incidence and milk types had a statistically significant difference (P <0.05).

Table 2. H. pylori incidence amongst examined k samples.

Types of raw milk samples	N. collected samples	N. samples positive for *H. pylori* (%)
Bovine	420	50 (11.90)
Ovine	380	92 (24.21)
Caprine	400	68 (17.00)
Total	1200	210 (17.50)

Genotypes distribution:

Table 3 expresses the H. pylori genotypes frequency amongst the isolates. VacA s1a (64.76%), s2 (57.14%), m1a (56.19%), and m2 (54.28%), cagA (49.52%) and cagE (49.52%) were the most frequently determined genotypes. IceA2 (10.47%), oipA (14.76%), and vacA s1c (15.23%) and m1b (15.23%) had the lowest distribution. The highest distribution of cagE genotype (92.00%) was found in the H. pylori isolates of raw bovine milk samples. H. pylori isolates of raw ovine milk samples harbored the highest distribution of other examined genotypes. Genotypes incidence were statistically significant amid dissimilar raw milk samples (P <0.05). Additionally, iceA1 and iceA2 incidences were statistically significant (P <0.05). Similarly, cagA and cagE A incidences were statistically significant (P <0.05).

Table 3. H. pylori genotype distribution.

Types of raw milk samples (N. positive)	N. isolates harbored each genotype (%)											
	VacA							*cagA*	*cagE*	*iceA*		*oipA*
	s1a	*s1b*	*s1c*	*s2*	*m1a*	*m1b*	*m2*			*IceA1*	*IceA2*	
Bovine (50)	30 (60.00)	10 (20.00)	5 (10.00)	25 (50.00)	23 (46.00)	6 (12.00)	24 (48.00)	19 (38.00)	46 (92.00)	7 (14.00)	2 (4.00)	3 (6.00)
Ovine (92)	65 (70.65)	19 (20.65)	17 (18.47)	60 (65.21)	61 (66.30)	15 (16.30)	57 (61.95)	58 (63.04)	36 (39.13)	27 (29.34)	14 (15.21)	20 (21.73)
Caprine (68)	41 (60.29)	14 (20.58)	10 (14.70)	35 (51.47)	34 (50.00)	11 (16.17)	33 (48.52)	27 (39.70)	22 (32.35)	14 (20.58)	6 (8.82)	8 (11.76)
Total (210)	136 (64.76)	43 (20.47)	32 (15.23)	120 (57.14)	118 (56.19)	32 (15.23)	114 (54.28)	104 (49.52)	104 (49.52)	48 (22.85)	22 (10.47)	31 (14.76)

DISCUSSION:

Milk (Angelidis et al. 2011; Rahimi and Kheirabadi 2012), meat (De Cooman et al. 2013; El Dairouty et al. 2016; Hamada et al. 2018), vegetables (Meng et al. 2008; Atapoor et al. 2014), salad (Atapoor et al. 2014), and ready-to-eat foods (Poms et al. 2001; Meng et al. 2008) were previously identified as *H. pylori* origins. The present research showed that the *H. pylori* incidence amongst raw milk samples was 17.50%. According to the literature searches, the *H. pylori* incidence among raw milk samples of naturally infected animal species had ranged between 1.00 to 35.00% (Quaglia et al. 2007; Quaglia et al. 2008; Angelidis et al. 2011; Fujimura et al. 2010; Rahimi and Kheirabadi 2012). We found that the *H. pylori* incidence amongst raw caprine, ovine, and bovine milk samples was 17.00%, 24.21%, and 11.90%, respectively. However, another Iranian survey (Talaei et al. 2015) reported the *H. pylori* incidence in raw caprine, ovine, and bovine milk samples as 4.76%, 13.79%, and 16.00%, respectively. Another research (Mousavi et al. 2014) revealed that the *H. pylori* incidence amongst raw bovine, ovine, and caprine milk samples was 16.66%, 35.00%, and 28.00%, respectively. *H. pylori* incidence in raw milk samples collected from Sudan (Bianchini et al. 2015), Italy (Bianchini et al. 2015), Greece (Angelidis et al. 2011), Japan (Fujimura et al. 2010), and Iran (Safaei et al. 2011) were 22.00%, 1.80%, 20.00%, 72.20%, and 40.00%, respectively.

Milk has a good capacity to harbor *H. pylori*, especially pH (4.9 to 6.0) and water activity (>0.97) (Atapoor et al. 2014; Gilani et al. 2017a). Additionally, Momtaz et al. (2014) (Momtaz et al. 2014) described the high *H. pylori* incidence in gastric biopsies of goat, sheep, and cow species. However, some additional research should be carried out to determine the exact source of the *H. pylori* presence in raw milk samples. Similar studies have been conducted by other researchers globally (Alihosseini et al. 2020; Mabeku et al. 2019; Alaali et al. 2020; Kipritci et al. 2020; Zou et al. 2020; Hunt et al. 2011; Mousavi et al. 2014; Hemmatinezhad et al. 2016; Ranjbar et al. 2019; Gilani et al. 2017b).

Genotyping pattern of isolates showed the high distribution of *m2*, *m1a*, *s2*, and *s1a*, alleles of the *vacA* gene amongst the *H. pylori* isolates. The high incidence of *vacA* genotypes was also documented in human clinical samples (Akeel et al. 2019; Ofori et al. 2019; Keikha et al. 2020). Our findings revealed that *cagA*-, *cagE*- and *oipA*- genotypes had a slightly higher distribution than *cagA*+, *cagE*+, and *oipA*+ amongst *H. pylori* isolates. However, the difference in distribution was significant only for between *oipA*+ and *oipA*- genotypes. Literature searches showed the gap in the knowledge about differences between *oipA*+ and *oipA*- genotypes of *H. pylori*. However, as the *oipA* gene is responsible for intracellular signaling and subsequent gastric inflammation (Teymournejad et al. 2017), its presence in the *H. pylori* strains implies higher severity. *H. pylori* isolates of raw bovine milk samples harbored the highest *cagE* genotype distribution (92.00%). Other genotypes had a higher distribution amongst the *H. pylori* isolates of raw ovine milk samples. *S2m2*, *s1am2*, *s1am1a*, and *s2m1a*, were the most frequently determined mutual genotypes. Similar reports are available about the genotyping of *H. pylori* isolates of raw milk samples. Khaji et al. (2017) (Khaji et al. 2017) stated that the frequency of *s1am1a*, *s2m1a*, *s1am2*, and *s2m2* in the *H. pylori* isolates of raw milk samples was 41.66%, 25.00%, 16.66%, and 13.33%which was similar to our report. Similarly, Mashak et al. (2020) (Mashak et al. 2020) testified that *vacA m1a* (50%), *s2* (76.92%), *s1a* (84.61%), and *m2* (39.13%), and *cagA* (55.76%) and *iceA1* (38.46%) were the most frequently determined genotypes amongst the *H. pylori* strains diagnosed in raw meat samples. High frequency of *s1am2* (51.92%), *s1am1a* (63.46%), *s2m1a* (53.84%), and *s2m2* (42.30%) was also determined in their survey. Talimkhani and Mashak (2017) (Talimkhani and Mashak 2017) stated differences in the genotyping profiles of *H. pylori* isolates of raw milk, retail meat, and raw vegetable samples. *VacA s2* (83.33%), *s1a* (87.50%), *m2* (62.50%), *m1a* (87.50%), and *cagA* (83.33%) were predominant amongst raw milk samples in their report.

High incidence of *iceA*, *vacA*, *oipA*, *cagA*, and *cagE* genotypes amongst the *H. pylori* isolates of food samples with animal origins were described by Gilani et al. (Gilani et al. 2017a,b), Hemmatinezhad et al. (2016) (Hemmatinezhad et al. 2016), Ranjbar et al. (2018) (Ranjbar et al. 2018), Ghorabani et al. (2016) (Ghorabani et al. 2016), and Mousavi et al. (2014) (Mousavi et al. 2014). All works conducted in this field revealed some similarities and some differences in the *H. pylori* genotyping profiles between food samples with human clinical infections and within diverse food samples (Mousavi et al. 2014; Hemmatinezhad et al. 2016; Gilani et al. 2017a,b; Ranjbar et al. 2018; Ghorbani et al. 2019; Ranjbar et al. 2019). Our findings also described similarities in the *H. pylori* genotyping profile between different raw milk samples. This finding may show similar routes of their contamination. However, supplementary researches should be carried out to found the exact route of raw milk contamination.

The present survey was limited to a comprehensive assessment of *H. pylori* other genotypes and analysis of other raw milk and dairy product samples. However, high numbers of collected samples and extensive

antibiotic resistance assessment were our survey's strength points.

CONCLUSION:

In conclusion, we documented many *H. pylori* isolates of raw milk samples of naturally infected caprine, ovine, and bovine species. The high *H. pylori* incidence amongst raw milk samples typifies that they may be the bacteria's natural source with a high ability to spread in the environment. Besides, most isolates harbored *cagA*, *vacA*, *iceA*, *cagE*, and *oipA* genotypes together. These findings may pose a potential role of raw bovine, ovine, and caprine milk samples as virulent *H. pylori* reservoirs. Consuming these kinds of milk in a raw form may cause bacterial transmission to the human population and subsequent gastrointestinal disorders. Higher distribution of *iceA1* than *iceA2*, *oipA*- than *oipA*+, *cagA*- than *cagA*+, and *cagE*- than *cagE*+ was another novel finding of the present study. Similarities in the *H. pylori* genotyping patterns found among diverse raw milk samples may represent their same contamination route. Some antibiotic agents did not show significant effects against *H. pylori* isolates. Further multifactorial investigations are essential to distinguish the relationship between *H. pylori* genotyping profile and antibiotic resistance in raw milk samples. Additionally, it is significant to clear the *H. pylori* zoonotic aspects in the future.

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