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Helicobacter pylori genotyping among patients referred to hospitals of Tehran using PCR-RFLP technique

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Abstract

Background: *Helicobacter pylori (H. pylori),* infecting half of the global population, undergoes genetic diversity. The aim of this study included *H. pylori* genotyping among patients referred to hospitals of Tehran using PCR-RFLP technique using *ureAB* and *ureCD* genes.

Materials and methods: Identifying and genotyping such prevalent and variable pathogens is challenging. To date, two versions of *H. pylori* have been sequenced, indicating an immense variability in its genome. To distinguish the *H. pylori* subtypes, the PCR-RFLP technique was used as a differentiation method. Among 37 *H. pylori-positive* gastric biopsies and 33 viable PCR products, *ureAB* and *ureCD* genes were selected for PCR-RFLP using *HaeIII* and *NdeII,* respectively. The resultant gel electrophoresis banding patterns were analyzed with visual evaluation.

Results: PCR-RFLP standard run results were more appropriate compared to a single of each technique where a pair of *H. pylori* strains outlined identical banding patterns considering *UreAB/HaeIII* and *UreCD/NdeII* runs.

Conclusion: The findings permit clinicians to make precise judgments on the priority of patients' treatment, cost reduction, and mitigation of the trend of resistance emergence.

Keywords: *Helicobacter pylori*, Genotyping, PCR-RFLP technique

Introduction

Due to the advances and developments in the field of bacteriology, new avenues were opened for research [1]. *Helicobacter pylori* (*H. pylori*) is a gram-negative curved bacillus that has infected half of the world (4.4 billion individuals) [2]. More importantly, *H. pylori* pathogen elimination has not been successful [3]. This bacterium allows researchers to accurately study the interaction between the human body and the host. This bacterium exists in 30 to 90 percent of the population, depending on socioeconomic conditions, and has been determined in all countries until now, while developing countries bear a more significant burden [4]. Because this bacterium is associated with diseases such as duodenal ulcer, chronic active gastritis [5], gastric cancer [6], esophagus cancer [7] and lymphoma [7] and in some cases heart disease [8], the diet differential treatments are applied to eradicate *H. pylori* around the world. Owing to the difference in drug availability and antimicrobial resistance of the bacterium, the standard treatment regimen of each region and country is different, posing problems for human health. It is worth mentioning that this bacterium can move between two forms depending on the required physiological activity, such as surviving in adverse environmental conditions [9 - 13].

H. pylori produces a high amount of urease enzyme, which is initially intracellular and then secreted following the autolysis. Following colonization onto the gastric mucosa, as a result of the death of several isolates, urease, and other outer membrane proteins are released and spread in the environment [10], and the conversion of urea into ammonia in the tissue surrounding the bacteria can neutralize the gastric acid, in which *H. pylori* can survive.

The common factors increasing *H. pylori* infection include the individual's social and economic status and familial history, while the effect of gender and race is controversial. The transmission routes have been well studied, but no comprehensive result has been achieved. The contamination by animals is discussed. Using PCR, the presence of *H. pylori* in water, food, and soil has been proven, but bacterial culture has not been possible in these cases. Therefore, it can be concluded that this bacterium is transmitted through the gastro-oral route. The method used in this study included gene amplification using PCR (Polymerase Chain Reaction) and then using RFLP (Restriction fragment length polymorphism). In 1996, the findings of PCR-RFLP were more accurate than those of the RAPD (Random Amplification of Polymorphic DNA)

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technique.

In 1999, a researcher named Burucoa [14] performed a combined analysis using the results of experimental and raw data from articles in the meta-analysis of PCR-RFLP, ribotyping, and RAPD, according to the power of differentiation and type recognition, and the ability to analyzing the results with PCR-RFLP succeeded in obtaining more acceptable and less ambiguous results. For this reason, this technique is proper for the isolation and identification of *H. pylori*. PCR-RFLP is a technique in which a genome fragment is targeted and amplified using PCR and treated with restriction enzymes in the following stages of analysis [15].

Considering *H. pylori* is a genetically diverse bacterium [16], the accuracy of the genotyping techniques needs to be addressed. We required additional typing methods in case of similar banding patterns in the PCR-RFLP technique among isolates with different toxicity effects. If the DNA fingerprint obtained from one gene does not allow the differentiation between species, its combination with the DNA fingerprint of another gene will increase the differentiation between bacterial species. This study aimed to genotype *H. pylori* species from a genetic point of view in gastric biopsy samples using the PCR-RFLP technique using two separate genes.

Materials and methods

This study was conducted from September to December 2013 on 37 patients aged 29-45 with gastrointestinal discomfort, and heartburn referred to the endoscopy department of Shahada Tajrish and Taleghani hospitals in Tehran. The gastric tissue biopsy samples (two samples from the antrum area) were taken. One of these samples was used for the rapid urease detection test or RUT. In this test, the intense activity of *H. pylori* urease in the medium containing urea and pH indicator causes the production of ammonia and rapidly changes the color of the medium. Another sample, due to the sensitivity of *H. pylori* to oxygen and environmental conditions, inside a 1.5 mL microtube containing a suitable transport medium (Brucella Broth transport medium) and onto the ice at 0 to 4°C in less than six months, was transferred to the laboratory for cultivation.
Extraction and isolation of *H. pylori* DNA by phenol-chloroform method: In this step, by lysing the bacteria, their contents were removed, and after separating its proteins, the DNA was precipitated. For this purpose, first, the bacteria kept at -80°C were cultured on Brucella agar medium containing 7% defibrinated sheep blood, and after three days of incubation, the resulting isolates were used for DNA extraction. A manual method was used for DNA extraction. After the detection of DNA load, the solution was kept at -20°C until used for PCR.

Gene amplification using PCR

One of the methods of typing and subtyping based on DNA is the use of the PCR technique. In this method, the desired primers are bound explicitly to particular gene loci, amplifying the fragment internal sequence between both primers. In this study, *ureAB* and *ureCD* were selected as target genes. The specific primers have been inferred in Table 1.

Table 1. The specific primers used for the amplification of *ureAB* and *ureCD* genes

Gene	Primer sequence	Amplicon size
ureA-ureB	FW(5' AGG AGA ATG AGA TGA ATA AG3') RV(5' TTG AAT GAA GAT GTT AAG G 3')	3650 bp
ureC-ureD	FW(5' GCA GTC TTT TTA CCA GCT CTC 3') RV(5' TGG GAC TGA TGG CGT GAG G 3')	2300 bp

Amplification of the *ureAB* gene in a final volume of 100 µL using 2.5 µL of template DNA extracted from *H. pylori*, 2 µL of forward primer and 2 µL of reverse primer (100 nanomol/mL), 2 µL of dNTP (containing each nucleotide with a concentration of 10 mM), 2 µL of Taq polymerase enzyme (1 U/µL), 8 µL of MgCl2 (25 mM), 10 µL of buffer (25 mM, 5/ 71 µL of sterile, nuclease-free distilled water was used. For UreCD gene amplification, 4 µL of MgCl2 was optimal, and in this case, 75.5 µL of nuclease-free distilled water was needed to bring the total volume to 100 µL. The thermal conditions for each primer pair included:
Protocol of PCR cycles to amplify the *ureAB* gene included 95 °C for 5 min (to denature the template), then 35 processes (95 °C for 45 s, 47 °C for 35 s, 72 °C for 3 min, then 72 °C for 10 min.
Protocol of PCR cycles to amplify the *ureCD* gene included 95 °C for 5 min (to denature the template), then 95 °C for 45 s, 60 °C for 35 s, 72 °C for 3 min, then 72 °C for 10 min. The entire process followed the sterile protocol and considered the negative and positive control. To check the results, 15 µL of the reaction mixture was electrophoresed on 1.2% agarose gel.

Extraction and purification of target genes from gel electrophoresis: QIAamp Gel Extraction kit was used to extract DNA from the gel. After extraction, the DNA was used for enzymatic digestion reactions.

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Enzymatic digestion of the resulting DNA using restriction enzymes

The *ureAB* gene was digested by 1 µL of HaeIII. The resulting mixture was placed in a thermoblock overnight at 37°C. Then, the electrophoresis was performed using 2% agarose gel to separate the bands resulting from enzymatic digestion.
The *ureCD* gene was digested with 0.5 µL of NdeII and 10 µL of particular enzyme buffer, plus 10 µL of the PCR product, and the procedure was continued as described for that of *ureAB* gene.

Electrophoresis of the products or fragments

All the figures prepared were analyzed in the enzymatic digestion stage. The size of the bands was evaluated with the size of the corresponding banding pattern from control DNA.

Results

Out of 37 kept biopsy samples, bacterial culture thrived in 33 samples, and four were resistant to culture. PCR and enzymatic digestion for both *ureAB* and *ureCD* genes using HaeIII and NdeII enzymes, respectively, were successful.
Enzymatic digestion of *ureAB* by HaeIII gave 32 banding patterns with different sizes, of which at least two or more bands were observed in each patient, which correctly indicated the specific pattern of that patient on the gel. In other words, this method could separate all patients to determine the *ureAB* genotype, which inferred the difference of these strains at the DNA level, indicating the existence of different subspecies of *H. pylori* at the genetic level. In the bands created by *ureAB*/HaeIII enzyme digestion, a fragment with a length of 530 bp was observed in almost all patients, but fragments with a length of more or less than 530 bp had a lower frequency.
Enzymatic digestion of ureCD by NdeII could have been more informative and gave more than 15 banding patterns of different sizes. There was a more remarkable banding similarity where many subspecies had the same banding pattern as in patients 11, 18, and 31. Subspecies of this bacterium were detected. Also, another subspecies was identified in patients with numbers 8, 9, 10 and 25. Another subspecies was found in patients with numbers 16, 17, and 26. In addition, in patients with numbers 2, 3, 4, 5, 14, 15, 22, 23, 24, 27, 28, and 29, bands with the same length were observed in *ureCD*/NdeII enzymatic digestion, and only in patients with numbers 12, 20, 13, 19, 10, 6, 21, 30, 32 and 33 different banding patterns were created using this enzyme digestion, which is listed in Tables 9 and 10.

Generally, the pattern of bands observed on the agarose gel in these patients was more specific for *ureAB*/HaeIII enzymatic digestion than *ureCD*/NdeII enzymatic digestion. In this way, the difference in bacterial genotype was more evident despite the phenotypic similarity. When cost and time are discussed, and single enzyme digestion is considered, using ureAB/HaeIII enzyme digestion is preferable to UreCD/NdeII enzyme digestion.

Although UreAB/HaeIII enzymatic digestion produced specific results for the detection of *H. pylori* subspecies, combining the effects of both UreAB/HaeIII and UreCD/NdeII enzymatic digestion provides more detailed and accurate differentiation of genotypes [13] and banding patterns. More distinct or differentiation of DNA fragments on gel electrophoresis facilitates more specific detection of subspecies by removing the similarity of banding patterns in similar cases, using the same enzyme, thus resulting in more accurate differentiation of subspecies.

In this research, the combination of PCR and RFLP methods was used, and the PCR technique was used to amplify *ureAB* and *ureCD* genes. The combination of PCR-RFLP in the form of two enzymes created the maximum differentiation between *H. pylori* subspecies; thus, all subspecies were recognized and separated.

Regarding *ureAB*/HaeIII as well as *ureCD*/NdeII, the total number of bases in several subspecies was smaller than the expected size, which indicates the deletion in several subspecies or due to successive cuts and the production of tiny fragments which are not detected during electrophoresis.
Figures 1-8 and tables 2-11 have represented PCR-RFLP results for two *ureAB*/HaeIII and *ureCD*/NdeII genes after enzymatic digestion of PCR products of isolates from 33 examined patients.

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Table 2. The bands from PCR-RFLP for *ureAB*/HaeIII for each sample, for sample numbers 1 to 10.

No→	1	2	3	4	5	6	7	8	9	10
230										
250				*	*					
260			*							
290										
300	*									
310										
320										
330			*				*	*		*
340				*						
390	*			*						
400										
450	*	*		*						
460										
470			*	*				*		
480							*			*
530	*	*			*	*		*		*
550							*	*		
600				*				*		*
630										
640										
690		*								
700										
790	*	*				*				
800			*						*	
830										
900										
1100						*	*			*
1250										
1300										
1350										
1600					*					
1700									*	
Sum	2460	2460	1860	2500	2380	2420	2460	2480	2500	3040

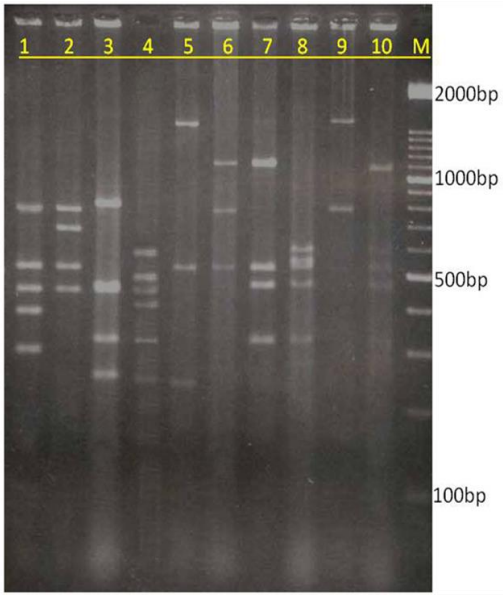


Figure 1. Banding pattern following PCR-RFLP for *UreAB*/HaeIII for samples 1-10.

Table 3. The bands from PCR-RFLP for *ureAB/HaeIII* for each sample,including samples 11-16.

No→	11	12	13	14	15	16
230				*		
250						
260						
290						
300	*	*			*	*
310						
320	*	*		*	*	*
330			*			
340						
390						
400					*	
450					*	
460						
470			*			
480	*			*	*	*
530	*	*	*	*	*	*
550						
600						
630						
640						
690						
700						
790						
800						
830	*			*		*
900						
1100			*			
1250						
1300		*				
1350						
1600						
1700						
Sum	2460	2450	2430	2390	2480	2460



Figure 2. The band's position or size resulted from PCR-RFLP for *UreAB/.HaeIII* for each sample, including samples 11-16 .

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Table 4. The bands from PCR-RFLP for *ureAB|HaeIII* for each sample, for PCR-RFLP for *ureAB|HaeIII*, and their sizes for samples 17-23.

No→	17	18	19	20	21	22	23
230							
250							
260				*			
290							
300			*	*	*	*	
310							
320	*	*					
330			*			*	*
340							
390			*				
400					*	*	
450	*	*		*		*	*
460							
470	*	*				*	*
480							
530	*	*	*	*	*	*	*
550							
600							
630	*	*					
640							
690							*
700							
790				*			
800					*		
830							
900			*				
1100							
1250							
1300							
1350							
1600					*		
1700							
Sum	2400	2400	2450	2330	3630	2480	2470

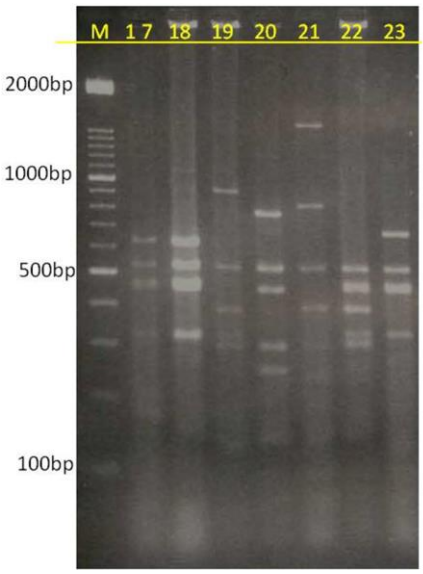


Figure 3. The banding pattern from PCR-RFLP for *UreAB/HaeIII* for each sample, considering samples 17-23.

Table 5. The band's position from PCR-RFLP for *UreAB/HaeIII* and total bands for each sample considering samples 24-33.

No→	24	25	26	27	28	29	30	31	32	33
230										*
250										
260						*				
290										*
300	*							*		
310		*								
320								*		
330		*								
340			*	*		*				
390							*			
400		*				*				
450		*		*						
460						*	*			
470								*		
480			*	*		*				
530	*	*	*	*	*	*	*	*		*
550			*						*	
600			*							
630										
640				*						
690										
700					*					
790	*						*			
800										
830	*							*		*
900										
1100										
1250					*					
1300										
1350									*	
1600										
1700										*
Sum	2450	2020	2500	2440	2480	2470	2170	2450	1900	3580

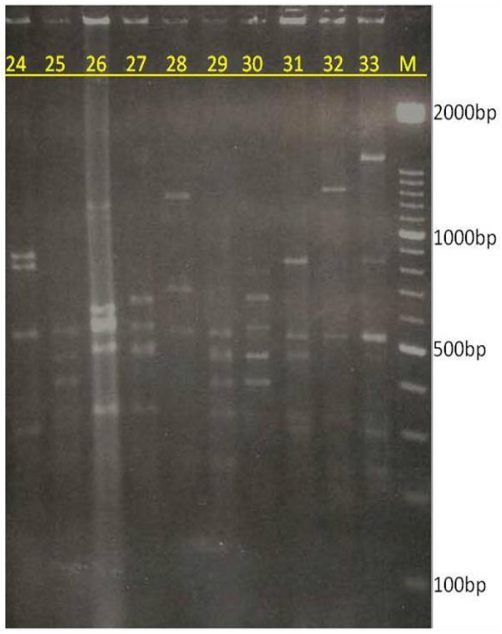


Figure 4. The bands from PCR-RFLP for *UreAB/HaeIII* for each sample considering samples 24-33.

Considering different and dissimilar banding pattern from PCR-RFLP using *UreAB/HaeIII*, none of 33 H. pylori isolates exhibited the same genotype highlighting various strains of the species.

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Table 6. The banding pattern from PCR-RFLP for *UreCD/NdeII* for samples 1-10.

No→	1	2	3	4	5	6	7	8	9	10
80	*									
160										
180	*	*	*	*	*	*	*	*	*	*
200										
230										
270										
300										
320										
340	*	*	*	*	*	*	*	*	*	*
450										
510						*		*	*	*
530	*	*	*	*	*	*	*	*	*	*
620	*									
700		*	*	*	*	*	*			
1220										
Sum	1750	1750	1750	1750	1750	2260	1750	1560	1560	1560

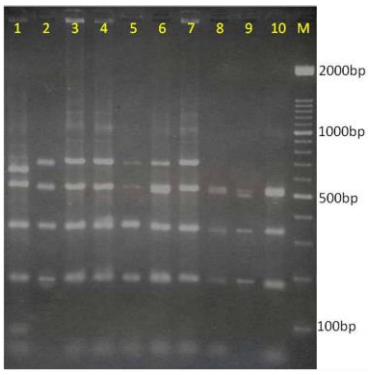


Figure 5. The banding pattern from PCR-RFLP for *UreCD/NdeII* for samples 1-10.

Table 7. The banding pattern from PCR-RFLP for *UreCD/NdeII* for samples 11-20.

No→	11	12	13	14	15	16	17	18	19	20
80										
160										
180	*	*	*	*	*	*	*	*	*	*
200						*	*		*	
230	*	*	*					*		*
270			*							
300	*	*	*					*	*	*
320						*	*			
340	*	*	*	*	*	*	*	*	*	*
450			*							
510										
530	*	*		*	*			*	*	
620										
700		*		*	*	*	*			*
1220										
Sum	1580	2280	1770	1750	1750	1740	1740	1580	1550	1750

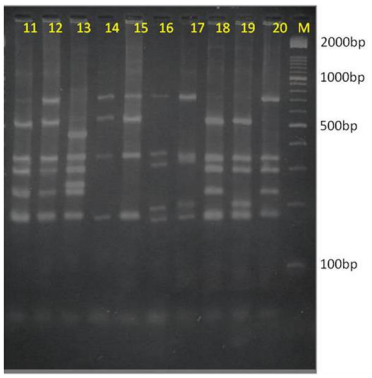


Figure 6. The banding pattern from PCR-RFLP for *UreCD/NdeII* for samples 11-20.

Table 8. The banding pattern from PCR-RFLP for *UreCD/NdeII* for samples 21-30.

No→	21	22	23	24	25	26	27	28	29	30
80										
160										*
180		*	*	*	*	*	*	*	*	*
200	*					*				
230										
270										
300										
320						*				
340	*	*	*	*	*	*	*	*	*	*
450										
510					*					
530	*	*	*	*	*		*	*	*	*
620										
700	*	*	*	*		*	*	*	*	
1220										
Sum	1770	1750	1750	1750	1560	1740	1750	1750	1750	1210

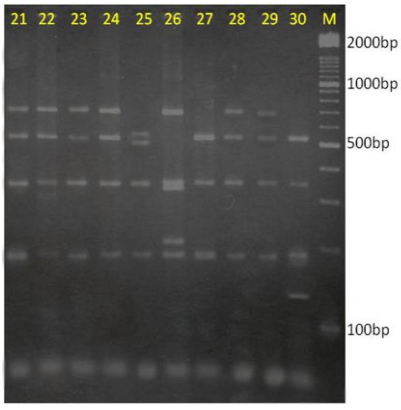


Figure 7. The banding pattern from PCR-RFLP for *ureCD*|NdeII for samples 21-30.

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Table 9. The results of PCR-RFLP using *UreCD/NdeII* for 33 isolates of H. pylori and genotypic relation of strains among 33 patients.

No→	31	32	33
80			
160			
180	*	*	*
200			*
230	*		
270			
300	*		
320			
340	*	*	*
450			
510			
530	*		
620			
700			*
1220		*	
Sum	1580	1740	1420

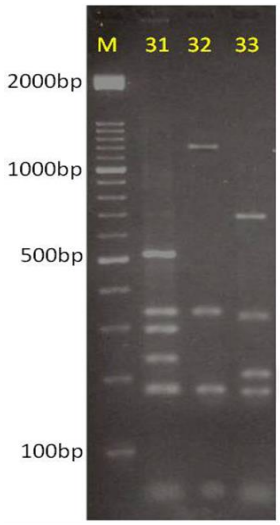


Figure 8. The banding pattern from PCR-RFLP for *UreCD/NdeII* for samples 31-33.

Considering the banding pattern of PCR-RFLP for *UreCD/NdeII* for 33 isolates of H. pylori, a similar pattern exhibited a relation of strains that demonstrated lower genetic diversity.

Table 10. The banding pattern from PCR-RFLP for *ureCD*/NdeII for 33 *H. pylori* isolates among patients

No→	11	18	31	8	9	10	25	16	17	26	2	3	4	5	7	14	15	22	23	24	27	28	29
80																							
160																							
180	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
200								*	*	*													
230	*	*	*																				
270																							
300	*	*	*																				
320								*	*	*													
340	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
450																							
510				*	*	*	*																
530	*	*	*	*	*	*	*				*	*	*	*	*	*	*	*	*	*	*	*	*
620																							
700								*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
1220																							
Sum	1580	1580	1580	1560	1560	1560	1560	1740	1740	1740	1750	1750	1750	1750	1750	1750	1750	1750	1750	1750	1750	1750	1750

As represented in Table 10, using PCR-RFLP and *UreCD/NdeII*, some banding patterns were similar among patient isolates, which could be considered in one genotype.

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Table 11. The banding pattern from PCR-RFLP for *UreCD/NdeII* for 33 H. pylori isolates among patients

No→	12	20	13	19	1	6	21	30	32	33
80					*					
160								*		
180	*	*	*	*	*	*		*	*	*
200				*			*			*
230	*	*	*							
270			*							
300	*	*	*	*						
320										
340	*	*	*	*	*	*	*	*	*	*
450			*							
510						*				
530	*			*	*	*	*	*		
620					*					
700	*	*				*	*			*
1220									*	
Sum	2280	1750	1770	1550	1750	2260	1770	1210	1740	1420

According to Table 11, PCR-RFLP using *UreCD/NdeII* demonstrated no similarity among *H. pylori* isolates and could not be classified in one genotype.

Discussion

H. pylori belongs to several genetically distinct populations exhibiting high genetic diversity [17]. Since the 21st century, *the H. pylori* rate has decreased in developed countries in the Western world, while it remains high in developing or newly developed countries. In most regions, family transmission is the primary mechanism of *H. pylori* spread. According to the information obtained from the meta-analysis of 2018, the study of 410,879 patients from 73 countries in 6 continents shows that the global prevalence of *H. pylori* infection is 44.3%.

This prevalence in developing countries has been reported as 50.8% and in developed countries as 34.7%. There is a vast difference in the prevalence of this infection among various regions and countries. The highest prevalence is related to Latin America, Africa (79.1%), the Caribbean (63.4%), and Asia (54.7%). While the lowest prevalence rate is reported in North America (37.1%) and Oceania (24.4%). The prevalence rate of this infection is also high in Iran and reaches 50.7%, and the highest and lowest prevalence rate of this infection is in the order related to the provinces of Tehran (74.27%) and Mazandaran (19.2%) [18].

This difference in *H. pylori* prevalence can reflect urbanization, health, access to clean water, and changed socioeconomic levels. It has been observed that the socioeconomic level and higher education level are protective factors against contracting this infection. The success of this research is that almost every patient sample can be distinguished into a particular subspecies using two gene loci in PCR and a specific and individual enzyme digestion protocol, which can be seen with gel electrophoresis. The use of PCR or Enzyme digestion is not singly effective, and as described in the results section, the combination of the two provides better results. Because all patients were unrelated, *H. pylori* diversity was expected to be more significant. As mentioned in the results section, some of these bands were smaller than expected.

Another explanation for the size difference could be double bands, where two fragments of approximately equal size appear on the gel and overlap each other, and the band is thicker and brighter than usual, indicating difficulty in the experiments. Colonization of patients with more than one subspecies of *H. pylori* among Europeans and North Americans seems rare. Taylor, in 1995, found more than one colonizing subspecies in only three out of 15 patients, and Marshall, in 1996, found only one subspecies in 19 patients from two infecting species. In developing countries such as Peru, 34% has been reported [19].

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In an article published in Science by Daniel Falush et al. [20] in 2003, 370 subspecies were collected from various ethnic groups in different regions using sequencing studies of seven housekeeping genes. The toxicity of species was related to the *vacA* gene, and using a computerized program called structure, providing the relationship between the history of human migration and the prevalence of *H. pylori*, they analyzed all the sequenced strains and classified them into four new populations. After grouping, these four groups were re-divided into seven prominent people. It can be proven that *H. pylori* has been related to humans since the distant past, and the relationship between species is consistent with human migration from one continent to another and even includes the exchange of slaves from Africa to America and Europe [21,22].

This research should be interpreted along with a significant examination of the nature of *H. pylori*. This study proved that combining multiple PCR-RFLP for several genes is preferable to the single-gene protocol performed on one species. Breaking the subspecies of a group by the second PCR-RFLP, namely UreAB/HaeIII, into new groups or specific groups significantly leads to higher interspecies differentiation. As a result, the diagnosis of *H. pylori* should not be based on using only one differentiation method. Using an ideal combination of tests makes it possible to differentiate between the two subspecies of *H. pylori*.

If a database for the genotype of the known subspecies and their phenotype is provided for these tests, the diagnosis of *H. pylori* subspecies will be better, leading to the accurate prescription of antibiotics reducing drug resistance and treatment costs. Therefore, the data provides an effective treatment method for the patient population. *H. pylori* is one of the most common bacterial pathogens in the human body and is prevalent worldwide. Its presence and relationship with common and important diseases have been demonstrated. Research on this pathogen must be continued to ensure the successful management of infection elimination.

More importantly, since the treatment with the widespread use of antibiotics cannot be effective, sooner or later, other new treatment methods will be developed to deal with this drug-resistant bacterium. The affordability of new antibiotics is not possible in the assay. A clearer understanding of which organisms exert specific effects on the human body is valuable for progress in this field. DNA fingerprinting using PCR-RFLP is a suitable and reproducible method for distinguishing species and establishing the relationship between phenotype and genotype, as shown in many studies. Determining the genotype of *H. pylori* subspecies and classifying them based on toxicity gives physicians the authority to eradicate *H. pylori* infection based on their standard method. In this approach, those toxic subspecies can be specifically targeted using antibiotics to reduce antibiotic resistance. Therefore, a list of poisonous subspecies for each population or race should be prepared.

Conclusion

This study assessed a low number of *H. pylori* isolates regarding genetic relation. A different and dissimilar banding pattern was observed in PCR-RFLP using *ureAB*/HaeIII, where none of the 33 *H. pylori* separates exhibited the same genotype. In addition, considering the banding pattern of PCR-RFLP using *ureCD*/NdeII for 33 isolates of *H. pylori*, more similar patterns or more relation of strains demonstrated lower genetic diversity. It was shown that PCR-RFLP can distinguish strain genotypes, though more high-troghouput techniques are required.

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Authors' contributions

S.B. supervised the study, D.E. advised the study. H.A. performed the work and wrote the study. A.G. wrote and edited the draft.

Conflicts of Interest

The authors stated that there is no conflict of interest.

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