



Original Article

# Molecular detection of *Helicobacter pylori* based on the presence of *cagA* and *vacA* virulence genes in dental plaque from patients with periodontitis



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## KEYWORDS

*Helicobacter pylori*;  
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**Abstract** *Background/purpose:* *Helicobacter pylori* (*H. pylori*) infection is the most common in the world and is associated with various gastrointestinal pathologies, including chronic gastritis, peptic ulcers, and gastric cancer. The prevalence is associated with socioeconomic conditions, with this infection being more common in developing countries than in developed countries. The presence and permanence of *H. pylori* in the oral cavity has been reported, but its role is controversial. The aim of this study was to determine the prevalence of *H. pylori* in dental plaque of patients with periodontitis.

*Materials and methods:* A cross-sectional study was carried out and Periodontal Screening and Recording (PSR) index was determined. 38 dental plaque samples were taken and total DNA was extracted and qPCR was performed.

*Results:* 60.5% of the samples ( $n = 23$ ) were positive for the presence of *H. pylori* by the amplification of the 16S rRNA and *vacA* genes. In addition, *cagA* gene was detected in 21.7% ( $n = 5$ ) of *H. pylori*-positive. A significant relationship between periodontal status and *H. pylori* oral

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infection was found ( $P \leq 0.05$ ); patients with initial and moderate periodontitis were the most affected with 39.1% and 30.4%, respectively.

**Conclusion:** Our results suggest that the prevalence of *H. pylori* in the oral cavity could be related to the progression of periodontal disease. Therefore, oral hygiene and treatment for the elimination of oral *H. pylori* could stop the progression of periodontal disease.

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## Introduction

*Helicobacter pylori* (*H. pylori*) belongs to the subdivision of Proteobacteria, order *Campylobacter*, of the family *Helicobacter* and 20 species are recognized. This microorganism has the property of being microaerophilic and of being catalase, oxidase, and urease positive in most cases.<sup>1</sup> The lineages in which we can find this species can be subdivided into gastric and hepatic enterococci *Helicobacter*; these show a high prevalence of being organ-specific, since gastric *Helicobacter* do not have the capacity to colonize intestine and liver.<sup>1,2</sup> *H. pylori* presents, a genome of  $1.66 \times 10^7$  bp included in a single circular chromosome and a G + C content of 39%, within the genome has five regions that have a different composition of G + C, in the which two possess one or more copies of IS605 insertion sequences, 5S ribosomal RNA sequences at the ends and a 521 bp duplication close to each other.<sup>3</sup>

*H. pylori* infection causes mainly gastritis and one of the main risk factors for developing gastric adenocarcinoma and MALT lymphoma, the development of these clinical pathologies is influenced by various aspects, genetic susceptibility of the host, the environment, and the factors of virulence of the infecting strain; this being one of the most important. Virulence factors are bacterial products or strategies that confer pathogenicity. *H. pylori* several virulence factors have been proposed, such as *cagA*, *vacA*, among others, none of them implies the development of a specific disease. Risk of developing some clinical pathology increases the more virulence factors the bacteria accumulate.<sup>4,5</sup>

CagA is a protein with an approximate weight of 140 kDa, is highly immunogenic and is encoded by the *cagA* gene which is present in western countries in 60–70% of the isolated bacteria. *H. pylori* strains associated with *cagA* are a genomic marker of the pathogenicity island (PAI).<sup>6</sup>

The most studied molecular target for *cagA* is SHP-2 phosphatase (tyrosine phosphatase protein). Mutations and polymorphisms related to gastric carcinogenesis have been found into coding gene.<sup>7</sup> Also, changes that occur in gene expression after *H. pylori* infection in epithelial cells are usually dependent on the secretion system encoded by *cag* PAI.<sup>8</sup>

On the other hand, in several studies showed that some strains of *H. pylori* produced a protein in the culture supernatant said proteins induced vacuolization in epithelial cells, cell death and the destruction of epithelial integrity by such was designated to the protein as cytotoxin vacuolizing (VacA).<sup>9</sup> VacA protein is encoded by the *vacA*

gene and is present in all strains of *H. pylori*; however, only 50% of the strains show detectable cytotoxic activity. This forms part of a multimeric complex of approximately 100 kD and presents at least two variable parts.<sup>10</sup> However, these present variations in their vacuolizing activity due to heterogeneity within the *vacA* gene.<sup>11</sup>

The VacA protein could induce programmed death, independently of vacuolization, since it induces the release of cytochrome C from mitochondria through proapoptotic Bax and Bak proteins. This participates in the process of apoptosis by the activation of the Fas/CD95 receptor, through caspase 3 and the breakdown of the mitochondrial membrane that alters the cell cycle by disturbing the cellular ATP concentration.<sup>12</sup>

Geographic location, ethnic group, socioeconomic status, and age are variables that directly influence the prevalence of *H. pylori*. Several studies have revealed that there are several ways in which this bacterium could be acquired, within these forms have been considered a route of gastro-oral, oral-fecal transmission that is believed to be the main form of transmission especially in underdeveloped countries.<sup>13</sup>

Several studies have evaluated dental plaque and saliva as possible samples for the diagnosis of *H. pylori* using various techniques. The culture of the bacteria from the oral cavity has seldom been successfully isolated. With the different molecular techniques, better results have been reported when using dental plaque and saliva as samples for the detection of *H. pylori*.<sup>14</sup> The oral cavity has been considered a reservoir of infection of this microorganism, in different studies have shown results has been assumed as a reservoir of *H. pylori* infection.

Most studies have shown the presence of *H. pylori* in the oral cavity from patients who show to have some periodontal complication, so the American Dental Association developed the Periodontal Screening and Recording (PSR) index to improve the detection of periodontal disease.<sup>15,16</sup> Presence of this bacterium in the oral cavity has been postulated as an important potential source of reinfection after it has been eradicated from stomach.<sup>17</sup> Bürgers et al.<sup>18</sup> in their research showed that the presence of *H. pylori* can occur in the oral cavity independently of colonization of the stomach.

Mexico has a high prevalence (70%) of the microorganism and severe pathologies associated with it, it is relevant the genotypic diagnosis of *H. pylori*, using the qPCR for the detection of virulence factors and thereby determine the circulating variants. In addition, it is important that it be

done with a reduced invasiveness to the patient, since usually, gastric biopsies are used for this purpose.

The aim of this study was to determine the prevalence of *H. pylori* in dental plaque of patients with periodontitis.

## Materials and methods

### Study and patients

A cross-sectional study was carried out. This study was submitted and approved by the Ethics Committee in Biological Science College at Universidad Autónoma de Nuevo León (FCB-UANL) and Centro de investigación y Desarrollo en Ciencias de la Salud (CIDICS-UANL).

Patients were attended at dental clinic in Esquipulas, Chiapas, Mexico, during April 2016 and selected according to the following exclusion criteria: 1) current history of antibiotic usage or during the previous six months, 2) had a systemic condition that required prophylactic antibiotics, 3) dyspeptic symptoms, 4) history of gastrointestinal diseases and 5) were pregnant.

The patients who accepted their participation in this study signed the informed consent form. After, the dental clinical history, PSR score by sextant and dental plaque samples were taken. All experiments on human subjects were conducted in accordance with the Declaration of Helsinki and all procedures were carried out with the adequate understanding and written consent of the subjects.

### Periodontal examination

PSR is a quick, reliable, and reproducible method for identifying patients that may require a more complete evaluation of their periodontal health status.<sup>19</sup> The plastic PSR Periodontal Examination Probe was used throughout this study. The probe has a 0.5 mm diameter ball tip and a color-coded band extending 3.5 mm–5.5 mm from the tip. The PSR probe was gently inserted into the gingival sulcus of each tooth until the light resistance was reached and then the probe was moved around the circumference of the tooth. The depth of the probe in each sextant of the mouth was determined and recorded. Intraoral sextants were designated S1–S6, beginning in the maxillary right sextant (S1), proceeding in a clockwise manner, and finishing in the mandibular right sextant (S6). Each sextant was assigned a code based on the highest probing value obtained on any tooth in that sextant.

PSR codes were based on the following system: 0) Colored area of probe remains completely visible in the deepest crevice in the sextant. No calculus or defective margins are detected. Gingival tissues are healthy with no

bleeding after gentle probing. 1) Colored area of probe remains completely visible in the deepest probing depth in the sextant. No calculus or margins are detected. There is bleeding after gentle probing. 2) Colored area of probe remains completely visible in the deepest probing depth in the sextant. Supra- or subgingival calculus and/or defective margins are detected. 3) Colored area of probe remains partly visible in the deepest probing depth in the sextant. 4) Colored area of probe completely disappears, indicating probing depth of greater than 5.5 mm.<sup>19,20</sup>

### Collection of dental plaque samples

Subgingival plaque samples were collected with the help of sterile curette after careful removal of supragingival plaque. In each subject, samples were collected from all posterior teeth. Subgingival plaque samples were suspended in 500 µL of 30 g/L TS broth (Becton Dickinson) supplemented with 30% (w/v) glycerol solution for the maintenance of bacterial viability. All samples were stored at –80 °C, until use.

### DNA isolation

For total DNA extraction, TRIzol reagent (Thermo Fisher Scientific, Waltham, MA) was used according to manufacturer's instructions. The concentration and purity of DNA samples, was analyzed in a NanoDrop 8000 UV–Vis spectrophotometer (Thermo Fisher Scientific). The 230/260 absorbance ratio of DNA samples was between 1.9 and 2.1. In addition, DNA samples were loaded in a 1% (w/v) agarose gel then stained with 0.5 µg/mL ethidium bromide solution and visualized under UV light.

### *H. pylori vacA* and *cagA* genes status and detection

*H. pylori* detection was performed by analysis of real-time PCR amplification (qPCR) using oligonucleotides targeting against to 16S rRNA gene. In addition, *vacA* and *cagA* genes status was analyzed by qPCR. The oligonucleotide sequence were previously designed in our laboratory by Urrutia-Baca et al.<sup>21</sup> as shown in Table 1. The qPCR reactions were performed in 96-well plates, as follows: SYBR qPCR master mix 2X (Thermo Fisher Scientific), 0.1 µM each primer, 40 ng of DNA sample and nuclease-free water to a final volume of 25 µL qPCR reactions was amplified in a LighCycler 480 thermal cycler (Roche, Basel, Switzerland), programmed with the Sybr Green I detection system, in four stages: i) pre-incubation: one denaturing cycle of 95 °C per 10 min; ii) Amplification: forty of amplify cycle (95 °C per 10 s and with a decrease of 4 °C/s, alignment at 55 °C for 10 s and decrease of 2 °C/s), one extension cycle at 72 °C per 10 s,

**Table 1** qPCR Primers used for identification and genotyping of *H. pylori*.

Gene	Forward	Reverse	Tm (°C)	Reference
16s rRNA	ggagtacggtcgcaagattaaa	ctagcggattctccaatgtaa	55	Urrutia-Baca et al., 2018
cagA	gaccgactcgatcaaatagca	ttagctgaaagccctacctac	55	
vacA	cctactgagaatggtggcaata	gttcttcacgagagcgtgatt	55	

with a decrease of 4 °C/s; iii) Dissociation curve: one cycle of 95 °C per 5 s with a decrease of 4 °C/s, at 65 °C for 1 min with a decrease of 2 °C/s and 97 °C with a decrease of 5 °C/s; and iv) *Cooling*: one cycle of 40 °C per 30 s and 2 s at 1.5 °C/s.

### Statistical analysis

The results were collected in a database using SPSS software v22.0 and the descriptive statistical analysis was performed. In order to determine the association between each nominal variable, Fisher's exact tests was applied; one-way ANOVA and post-hoc Tukey tests were used for numeric variables. The  $P < 0.05$  value was considered statistically significant.

## Results

### Description of patients

A total of 38 patients with periodontal disease, 4 males and 34 females ranging in age from 34 to 60 years with an average age of  $47.1 \pm 12.48$ , were included. In the periodontal examination of patients based on PSR index, showed that 39.5% of patients were diagnosed with moderate periodontitis, 34.2% with periodontitis initial, 10.5% with gingivitis and 15.8% with severe periodontitis (Table 2). In addition, 100% of male were diagnosed with moderate periodontitis.

### *H. pylori cagA* and *vacA* genes status and detection in dental plaque

*H. pylori* detection in the dental plaque samples collected from the patients was performed by qPCR assay. The qPCR amplification and melting curves obtained from each DNA sample were compared to DNA *H. pylori* J99 strain as control positive (Fig. 1). The results showed that 60.5% of the patients (23/38) were positive for the amplification of *H. pylori* 16S rRNA gene. Likewise, the *H. pylori vacA* and *cagA* genes were detected in 100% (23/23) and 21.7% (5/23) of *H. pylori*-positive patients. The mean values of cycle

threshold (Ct) obtained from each positive sample shown in Table 3.

### Relationship between periodontal status and *H. pylori* oral infection

A significant relationship was found between the periodontal status calculated by PSR and oral *H. pylori* infection using 16s rRNA and *vacA* genes ( $P < 0.05$ ). The 39.1%, 30.4% and 26.0% of the infected patients were found in the 2nd, 3rd and 4th classification of the PSR index, respectively. The PSR index describes 2nd, 3rd and 4th classification as initial, moderate and severe periodontitis, respectively (Table 4). On the other hand, *cagA* gene was detected in 21.7% 5/23 of *H. pylori*-positive patients and 40% of *cagA*-positive patients were within 3rd and 4th classification, both cases; moderate and severe periodontitis according to PSR examination, respectively (Table 5).

## Discussion

In Mexico, the seroprevalence of *H. pylori* has been reported to around 20% in children at one year of age, 50% at 10 years of age and the highest prevalence has been reported between 25 and 30 years of age where gastrointestinal pathologies related to this infection are also frequent. Almost always infected people may develop some clinical gastrointestinal pathology such as gastritis, peptic ulcer or gastric cancer.<sup>22</sup> Therefore, the study of the oral cavity is of great importance since it has been proposed as a native reservoir for *H. pylori* where reinfections after the eradication treatment against this bacterium.<sup>23</sup>

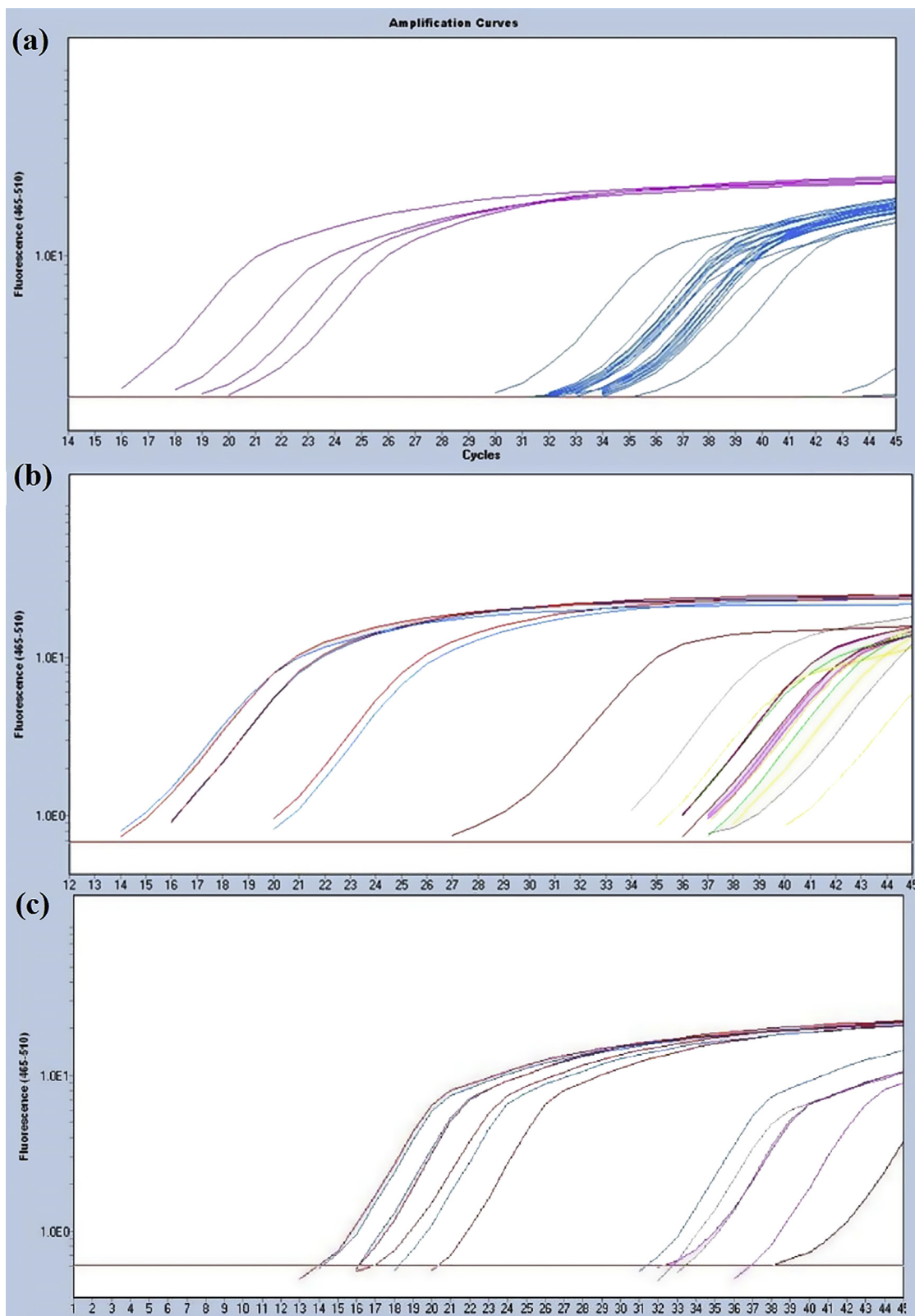
Kignel et al.<sup>14</sup> have suggested that the colonization of *H. pylori* in dental plaque does not go beyond some kind of local disease. However, in recent years a high prevalence of bacteria in this reservoir has been reported in patients with periodontal diseases. Several studies based on molecular techniques have been carried out to detect *H. pylori*, which show high sensitivity and specificity.<sup>24</sup> In the present study, we use the real-time PCR method that allows quantification, shorter diagnostic times and greater sensitivity for the diagnosis of *H. pylori*; the 16S rRNA gene was used for the identification of *H. pylori* and the virulence genes *vacA* and *cagA* for genotyping.

In our study we observed a high prevalence (60.5%) of oral *H. pylori* infection in patients with periodontal clinical manifestations. Our data were higher than those obtained by De la Garza-Ramos et al.<sup>23</sup> they reported 13% of oral infection per *H. pylori* in dental plaque samples from thirty Mexican patients using conventional PCR. Other studies have reported a prevalence of oral *H. pylori* around 38%, however those studies were carried out in asymptomatic Mexican children.<sup>24–26</sup>

Several studies have detected *H. pylori* in the oral cavity in both dental plaque and saliva.<sup>27</sup> It was demonstrated that the presence of oral *H. pylori* is related to the reappearance of gastric infection by *H. pylori* causing the failure of the therapies, and that the periodontal treatment and good oral hygiene in infected patients significantly increases the eradication of *H. pylori* in the stomach.<sup>28</sup> However, there is a lot of disagreement among

**Table 2** Distribution of patients by gender, age and periodontal status.

Variable	Patients N = 38
<b>Gender n (%)</b>	
Male	4 (10.6)
Female	34 (89.4)
<b>Age</b>	
Mean ( $\pm$ SD)	47.1 $\pm$ 12.48
<b>PSR classification n (%)</b>	
1	4 (10.5)
2	13 (34.2)
3	15 (39.5)
4	6 (15.8)



**Figure 1** Amplification curves for detection of *H. pylori* genes by qPCR. (a) *16S rRNA*, (b) *vacA* and (c) *cagA* genes.

researchers about the role of dental plaque as an extra-gastric reservoir in the transmission of *H. pylori*.<sup>29</sup> Also, the oral *H. pylori* infection has been related to periodontal problems.<sup>30,31</sup> Reports including clinical and experimental

studies have reported that people with *H. pylori* infection tend to periodontal disease.<sup>27,32</sup> However, some studies showed that there was no correlation between *H. pylori* infection and periodontal status.<sup>33</sup> In our study, PSR

**Table 3** Cycle threshold values for 16S rRNA, *vacA* and *cagA* genes by qPCR assay.

Patients	Cp 16S rRNA	Cp <i>vacA</i>	Cp <i>cagA</i>
P1	32.68	34.68	
P3	33.36	36.44	
P5	33.6	35.66	
P8	33.52	35.65	
P9	31.69	35.55	
P10	33.36	33.69	33.51
P11	35.03	34.65	36.91
P12	42.37	32.57	
P14	32.23	37.07	32.84
P17	31.41	35.52	38.03
P18	41.09	37.05	
P19	29.41	25.63	
P21	31.89	35.5	
P22	31.52	37.03	
P25	30.85	33.55	
P26	31.82	34.67	
P27	32.89	34.47	
P28	31.12	36.01	32.63
P30	32.89	34.52	
P32	33.65	32.5	
P33	32.67	35.5	
P35	33.5	32.67	
P36	31.67	35.35	

**Table 4** Relationship between the PSR classification and *H. pylori* detection.

PSR classification n (%)	<i>H. pylori</i> 16s rRNA status		P value
	Positive N = 23	Negative N = 15	
1	1 (4.3)	3 (20.0)	0.044 <sup>a</sup>
2	9 (39.1)	4 (26.7)	
3	7 (30.4)	8 (53.3)	
4	6 (26.0)	0	

<sup>a</sup> Exact Fisher test.

analysis showed that the majority of patients have moderate periodontitis (39.5%), followed by periodontitis in the initial phase (34.2%).

It is known that some *H. pylori* strains directly influence the severity of gastrointestinal disease in infected patients. It is reported that individuals infected with *cagA*-positive *H. pylori* strains have a higher risk of developing peptic ulcer and gastric cancer.<sup>34</sup> It is possible that the accumulation of periodontopathogenic bacteria, the permanence of pathogenic *H. pylori* strains and increased concentration of bacterial metabolites in the periodontal tissues could contribute to a chronic inflammatory process. Hu et al.<sup>35</sup> evaluated the correlation between *H. pylori* infection with periodontal parameters, periodontal pathogens and inflammation in 14 patients. They obtained frequencies of *Porphyromonas gingivalis*, *Prevotella intermedia*, *Fusobacterium nucleatum* and *Treponema denticola* significantly higher with *H. pylori* infection than those without *H. pylori* infection. In that same study, they observed an increase in the expression of periodontitis-related molecules Wnt5a, interleukin 8 (IL-8), interleukin 6 (IL-6) and interferon gamma (IFN- $\gamma$ ) in human acute monocytic leukemia cells (THP-1) stimulated with *cagA*-positive strain (*H. pylori* 26695).

In our study, a 21.7% (5/23) positivity was found for the *cagA* gene among the infected patients, however, a relationship of this gene with the periodontal state of the patients was not observed.

In this way we agree with what was published by Kignel et al.,<sup>14</sup> when it is stated that in previous studies the use of dental plaque has been achieved as a possible non-invasive sample for the diagnosis of *H. pylori*. Likewise, with the results obtained in this research. We agree with published studies since they imply that *H. pylori* is found as a reservoir in the oral cavity, especially in dental plaque, and that the real-time PCR technique (qPCR) is the most appropriate due to its high sensitivity and specificity in order to detect a low number of *H. pylori* bacteria and their virulence genes in non-invasive samples.<sup>15,23,24</sup>

Our results allow us to confirm that it is possible to detect the presence and circulating genotype of *H. pylori* by amplifying the 16s rRNA gene and their *cagA* and *vacA* virulence genes using qPCR in dental plaque in patients from Esquipulas, Mexico, since this has been a technique that shows satisfactory results in terms of sensitivity, specificity, low cost and easy processing, although of course, complementary studies with a greater number of samples would provide data with greater forcefulness.

qPCR has great advantages for those epidemiological studies in which we intend to study the prevalence of

**Table 5** The *cagA* gene status in *H. pylori*-positive patients by PSR classification.

PSR classification n (%)	<i>cagA</i> gene status in <i>H. pylori</i> -positive patients		P value
	Positive N = 5	Negative N = 18	
1	0	1 (5.6)	0.648 <sup>a</sup>
2	1 (20.0)	8 (44.4)	
3	2 (40.0)	5 (27.8)	
4	2 (40.0)	4 (22.3)	

<sup>a</sup> Exact Fisher test.

infection in the population and its relationship with different gastrointestinal pathologies. These procedures will allow the diagnosis to be extended in those patients who are asymptomatic or who have symptoms of some gastrointestinal pathology.

## Conflicts of interest

None declared.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jds.2019.01.010>.

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