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CLINICAL RESEARCH:

Molecular Detection of *Helicobacter pylori* in Subgingival Biofilm of Patients with Periodontal Disease: A Cross-Sectional Study

Detección molecular de *Helicobacter pylori* en biofilm subgingival de pacientes con enfermedad periodontal: estudio de corte trasversal

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ABSTRACT: This cross-sectional observational study aimed to determine the presence of *Helicobacter pylori* (*H. pylori*) in the subgingival biofilm (SB) of patients with periodontitis treated at the Clinic of Periodontics of the Faculty of Dentistry of the University of Costa Rica. Fifty-eight patients with periodontitis were recruited. *H. pylori* antigen detection in stool samples was performed using a qualitative rapid test. SB samples were collected from patients with positive stool results and cultured in Skirrow media, agar tubes, and urea broth under microaerophilic conditions. DNA was extracted and analyzed by polymerase chain reaction (PCR) for *H. pylori*, and for *Porphyromonas gingivalis* (*P. gingivalis*). Of 40 analyzed stool samples, 22 tested positive for *H. pylori*. Eighteen SB samples from these patients were analyzed. All were urease-positive. H. pylori was detected in all SB studied samples by PCR, colonies could not be isolated on Skirrow media. 16 were PCR-positive from SB, and 14 were PCR-positive from cultures. *P. gingivalis* was detected in 7 culture samples and 6 SB samples. Our findings suggest that *H. pylori* can colonize the subgingival biofilm of patients with periodontitis, as demonstrated through laboratory methods.

KEYWORDS: Dental plaque; Periodontitis; Subgingival biofilm; *Helicobacter pylori*, *Porphyromonas gingivalis*.

RESUMEN: Este estudio observacional de corte transversal tuvo como objetivo determinar la presencia de *Helicobacter pylori* (*H. pylori*) en el biofilm subgingival (BS) de pacientes con periodontitis atendidos en la Clínica de Periodoncia de la Facultad de Odontología de la Universidad de Costa Rica. Se reclutaron cincuenta y ocho pacientes con periodontitis. La detección del antígeno de *H. pylori* en muestras de heces se realizó mediante una prueba rápida cualitativa. Se recolectaron muestras de BS de los pacientes con resultados positivos en heces y se cultivaron en medios Skirrow, tubos con agar y caldo de urea bajo condiciones microaerofílicas. Se extrajo ADN y se analizó mediante la reacción en cadena de la polimerasa (PCR) para *H. pylori*, y PCR para *Porphyromonas gingivalis* (*P. gingivalis*). De las 40 muestras de heces analizadas, 22 resultaron positivas para *H. pylori*. Se analizaron 18 muestras de BS de estos pacientes. Todas fueron positivas para ureasa; 16 fueron positivas por PCR a partir de BS, y 14 fueron positivas por PCR a partir de cultivos. La electroforesis en gel de agarosa confirmó 15 muestras positivas por PCR. *P. gingivalis* se detectó en 7 muestras de cultivo y 6 muestras de BS. *H. pylori* se detectó en todas las muestras de BS mediante PCR. Sin embargo, no fue posible aislar colonias de *H. pylori* en el medio Skirrow. Nuestros hallazgos sugieren que *H. pylori* puede colonizar el biofilm subgingival de pacientes con periodontitis, como se demostró mediante métodos de laboratorio.

PALABRAS CLAVE: Placa dental; *Helicobacter pylori*; Periodontitis; *Porphyromonas gingivalis*; Biofilm subgingival.

INTRODUCTION

Periodontal disease is a prevalent inflammatory condition of infectious origin that affects the oral cavity. An epidemiological study conducted across care centers of the Costa Rican Social Security Fund reported a periodontal disease prevalence of 59.10% in adults aged 20-45 years, and 40.64% in the elderly population (1). Although over 200 systemic conditions-including smoking and diabetes mellitus-have been linked to periodontal disease, the primary etiological factor remains dental biofilm, notable for its diverse and abundant bacterial composition. Among the key pathogens, *Porphyromonas gingivalis* (*P. gingivalis*) has been strongly associated with disease progression (2). This microorganism plays a critical role in the initiation and maintenance of microbial dysbiosis within the periodontal environment (3).

Helicobacter pylori (*H. pylori*) infection has been significantly associated with periodontitis, as reported in several studies (4-6, 9). This Gram-negative bacterium colonizes the gastric mucosa and is a well-established etiological agent of chronic gastritis, peptic and duodenal ulcers, and gastric cancer (12,13). Transmission is thought to occur primarily during early childhood through direct contact with the saliva, vomitus, or feces of infected individuals (14). Globally, over 50% of the population is estimated to be infected with *H. pylori* (15). Although the exact prevalence in Costa Rica remains unclear, available data suggest high infection rates (16, 17), which correspond with the country's elevated incidence of gastric cancer and associated mortality (18).

The oral cavity has been proposed as a reservoir for *H. pylori*, potentially contributing to reinfection after successful gastric eradication (19, 20). Studies have also linked *H. pylori* to various oral conditions, including canker sores, squamous cell carcinoma, tongue irritation, halitosis, and periodontal disease (21, 22). Evidence indicates that individuals with periodontitis may have up to a 3.34-fold increased incidence of *H. pylori* infection compared to those with healthy periodontal tissues (4), thereby elevating their risk of gastric

infection associated with this bacterium (5). It has been suggested that *H. pylori* detected in dental plaque is a significant risk factor for recurrent gastric infection (7). This indicates that dental plaque and periodontal pockets not only serve as reservoirs but may also facilitate recurrence of *H. pylori*-associated gastric conditions (8). Consequently, periodontal treatment is recommended as an adjunct to gastric *H. pylori* therapy, offering potential short- and long-term benefits for bacterial eradication (8-10).

Given *H. pylori's* high prevalence in Costa Rica, its pathogenic potential, and its association with both oral and digestive diseases, accurate identification in oral samples, is essential for assessing the oral cavity as a potential reservoir. Determining the presence of *H. pylori* in subgingival biofilm (SB) may support the development of preventive strategies to limit its persistence in the oral cavity as reservoir. This could help reduce risk factors associated with periodontal disease and gastric pathologies. Therefore, the aim of this study was to detect the presence of *H. pylori* in SB samples from patients with periodontal disease treated at the Clinic of Periodontics of the Faculty of Dentistry of the University of Costa Rica. We hypothesized that the bacterium *H. pylori* may be present in SB of patients with periodontal disease, highlighting the potential role of oral health in the infection and reinfection dynamics of both gastric and oral sites.

MATERIALS AND METHODS

Fifty-eight patients were recruited from the Clinic of Periodontics of the Faculty of Dentistry of the University of Costa Rica. All participants met the following criteria: a periodontitis diagnosis, 18 years or older, and a positive fecal *H. pylori* antigen test. Patients who had received antibiotic therapy (amoxicillin, tetracycline, metronidazole, or clarithromycin) within the preceding two months were excluded. Each participant completed a questionnaire to ascertain socio-demographic characteristics, their history of *H. pylori* diagnosis, prior eradication treatment, and gastric pathologies. The University of Costa Rica Ethics Committee approved the procedure for collecting data and the informed consent form.

After signing the informed consent, patients received a bottle and instructions for collecting a stool sample. These samples were then processed using a qualitative rapid cassette test (RightSign brand, Yuhang, China) to detect *H. pylori* antigen. Of the 40 stool samples received, 22 tested positive for *H. pylori* antigen. However, only 18 subjects provided complete information, allowing them to continue with the study. The remaining patients withdrew due to personal reasons or uncontrolled systemic diseases.

Before starting periodontal treatment, subgingival biofilm samples were collected from index teeth of these 18 patients using sterile curettes. Each biofilm sample was immediately placed into 1.5 mL microtubes containing 500 µL of RPMI 1640 culture medium (Gibco, Thermo Fisher Scientific, Waltham, USA). These samples were then transported to laboratory, ensuring delivery within a maximum of two hours from collection. All subsequent culture tests and molecular analyses were conducted in the laboratories of the Cancer Epidemiology Program at INISA.

H. pylori culture. 25 µL of each collected biofilm sample were seeded in duplicate onto plates prepared with selective Skirrow medium (Columbia agar, 10% lysed horse blood and antimicrobial supplements). The prepared plates underwent incubation at 37 °C for a minimum of five days in a 2.5 L jar, utilizing a microaerophilic atmosphere generated by CampyGen (Oxoid, Waltham, USA) in a humidified CO2 incubator. Subsequently, bacterial colonies observed on the plates were harvested, suspended in 1 mL of BHI (brain-heart infusion) broth containing glycerol,

and cryopreserved at -80 °C awaiting molecular analysis (1).

For the presumptive diagnosis of *H. pylori* based on urease activity, 25 µL of each sample were inoculated into Christensen's Urea Agar, and 50 µL was added to Stuart's Urea Broth. These tubes were incubated at 37 °C for two hours, with visual inspections performed every 20 minutes. A positive result was defined by a color change from yellow to fuchsia red, following the protocol described by Rodriguez *et al*., 2012 (2). The remaining portion of each sample was subsequently frozen at -80 °C for storage.

For bacterial DNA extraction, 400 µL of frozen colony samples and 250 µL of frozen biofilm samples underwent the CTAB (cetyltrimethylammonium bromide) method. This established protocol included digestion with Proteinase K and SDS, followed by phenol/chloroform purification and isopropanol precipitation. The isolated DNA pellets were resuspended in 100 µL of molecular-grade water, and their concentration was determined using a Nanodrop One (ThermoFisher Scientific, Waltham, USA). Subsequently, DNA dilutions were adjusted to 50 ng/µL in preparation for molecular assays.

H. pylori and *P. gingivalis* detection. Both reactions were performed using conventional endpoint PCR in a Veriti® thermal cycler (Applied Biosystems, California).

The *H. pylori*-specific 16S ribosomal RNA gene was characterized following a modified protocol from Chong *et al*., 1996 (3). Reactions were performed in a volume reaction of 20 µL, using 200 ng of genomic DNA, 10 nmol of each primer (F- 5′-CTG GAG AGA CTA AGC CCT CC-3′ and R-5′-ATT ACT GAC GCT GAT TGT GC-3′; Macrogen Inc, Republic of Korea), 200 µM dNTPs, 0.5 U Dream Taq polymerase, and 1X Dream Taq Buffer (all ThermoFisher Scientific, Waltham, USA) used the profile: 95 °C for 5 min; 40 cycles of 95 °C for 1 min, 61.5 °C for 1 min, 72 °C for 1 min; and a final 72 °C for 5 min. *H. pylori* strain 7.13 DNA (kindly provided by Dr. Christine Varon, INSERM, Bordeaux University, France and Dr. Silvia Molina, INISA, UCR) was included as a positive control. PCR products were subsequently analyzed by 2% agarose gel electrophoresis for 1 hour at 100 V, anticipating a 110 bp amplicon.

Molecular characterization of *P. gingivalis* was performed following a modified protocol by Quintero *et al*., 2011 (4). Each 20 µL reaction contained 150 ng of DNA, 200 µM of dNTPs, 25 nmol of each primer (F- 5´-TGT AGA TGA CTG AAA ACC-3' and R 5'-ACG TCA TCC CCA CCT TCC TC-3; Macrogen Inc, Republic of Korea), 0.5 U of Dream Taq polymerase, and 1X Dream Taq Buffer (ThermoFisher Scientific) with the following profile: initial denaturation at 94 °C for 5 min; 40 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s; followed by a final extension at 72 °C for 10 min. *P. gingivalis* DNA (kindly donated by Dr. Luis Alberto Acuña, Faculty of Microbiology, UCR) served as a positive control. PCR products were analyzed by 2% agarose gel electrophoresis at 100 V for 1 hour, with an expected amplicon size of 197 bp.

Statistical analyses were performed using GraphPad Prism software version 10.4 (GraphPad Software Inc., USA). The Mann-Whitney U test was employed to evaluate the relationship between bacterial prevalence and clinical parameters. For comparing means, either Student's t-test or the Mann-Whitney U test was applied, depending on data distribution. In contingency tables, the chi-square test or Fisher's exact test was utilized. A p-value of less than 0.05 was considered statistically significant.

RESULTS

A total of 58 patients were recruited between May and September 2024; 36.21% were male and 63.79% were female. The average age of the sample was 50.02±13.98 years. Age distribution by sex was 51.86±14.99 years for men (20-70 years) and 48.89±13.42 years for women (22-79 years). No statistically significant difference was found between the mean ages of the two sexes (p=0.38).

Regarding other demographic characteristics, 5.7% of the participants reported having no formal education, 39.66% had completed primary education, 36.20% had completed secondary education, and 18.97% had completed university education. In terms of Body Mass Index (BMI), 39.65% of participants had a BMI over 29.9, 32.75% had a BMI between 25.1 and 29.9, 25.86% between 18.5 and 25, and 1.72% had a BMI below 18.5. Of the total sample, 4 patients were smokers, 10 reported a family history of gastric cancer, and 3 had been previously diagnosed with *H. pylori*.

Of the total recruited participants (n=58), only 40 stool samples were collected and analyzed for the detection of *H. pylori* at the gastric level. From this subset (n=40), a prevalence of 55% was observed, with a total of 22 patients testing positive for the antigen. Among the positive cases, 63.16% (n=12) were male and 47.62% (n=10) were female, indicating no significant difference in *H. pylori* prevalence between sexes (p=0.32).

Table 1 presents demographic and clinical characteristics of patients who tested positive for *H. pylori*. Of the 40 samples collected, 22 tested positive for the stool antigen test. Complete information necessary to continue with the study was obtained for only 18 patients. Of these, 44.4% were women and 54.6% were men. The mean age of this sample was 53.88±11.72 years. The age distribution by sex was 54.8±13.88 years for men and 52.75±9.11 years for women. No significant difference was found in the average ages between sexes (p=0.50). None of the positive patients were smokers, and 3 of the 18 patients reported a family history of gastric cancer.

Regarding the dental biofilm index (DBI) and bleeding index (BI), statistical analysis could not be performed due to the absence of two data points from two positive patients, that did not continue their treatment at the Clinic of Periodontics of the Faculty of Dentistry of the University of Costa Rica.

Among the participants who tested positive, periodontal disease diagnosis revealed that 9 individuals were classified as stage III, grade C; 4 as stage IV, grade C; 2 as stage III, grade B; 1 as stage IV, grade B; and 1 as stage II, grade B. Periodontal disease diagnosis was unavailable on the electronic health record for one participant due to discontinuation of treatment at the clinic. When periodontal disease stages were classified by age groups, the only participant under 50 years of age, was stage II. In the 51-60 age group, 3 participants were stage III and 3 stage IV. Among those over 60 years of age, 5 participants were stage III and 2 stage IV. These results indicate that the highest number of *H. pylori*-positive participants were over 60 years old and stage III. Based on these data, no significant association was found between age group and stage of periodontal disease (p=0.8586).

Of the 18 samples from patients who tested positive on the antigen test, all were positive for urease test, indicating urease activity. Also, all samples showed bacterial growth in culture. All cultured plates exhibited abundant growth of colony-forming units with diverse morphologies (Figure 1).

PCR was performed on the samples (n=18) of patients who tested positive on the antigen test, except for two samples, from which an insufficient amount of material was obtained for DNA

extraction. For the PCR tests in DNA obtained from biofilm, all analyzed samples were positive for *H. pylori*. In DNA samples isolated from culture, 14 were positive for *H. pylori*, while 4 were negative.

Additionally, PCR testing for *P. gingivalis* was performed on DNA extracted from cultures, yielding 7 positive results out of 18 samples (38.9%). PCR testing on DNA from biofilm showed 6 positive results. The results for *P. gingivalis* in biofilm and culture were consistent, except for one sample, for which not enough material was available for DNA extraction.

Table 2 compares data from 17 of the 18 patients who tested positive for the *H. pylori* antigen, as periodontal diagnostic information could not be obtained for one individual who did not continue treatment. According to the results, stage III was the most prevalent, with a total of 11 patients. Five patients were stage IV, and one patient was stage II. The age distribution for this population was 47 years for stage II patient, 55.09±7.92 years for those in stage III, and 59.40±9.29 years for those in stage IV. Five positive samples for *P. gingivalis* were found in stage III patients, and two in stage IV patients. However, there was no statistically significant association between bacterial presence and disease stage (p=0.99), nor between BMI and the presence of *P. gingivalis* (p=0.27), or between the BI and the presence of *P. gingivalis* (p=0.99). A statistically significant association was observed between the bleeding index and the presence of *P. gingivalis* (p=0.0073).

Table 1. Data for the *H. pylori*-positive population.

Patient	Age	Sex	DBI	BI	Stage	Grade
HP-001	43	Female	81,16	48,5	III	C
HP-002	43	Female	81,33	14,66	III	C
HP-003	60	Male	60,94	19,1	III	C
HP-004	60	Female	72,81	35	III	C
HP-005	70	Male	65	22	IV	B
HP-006	47	Female	76,67	15	II	B
HP-007	45	Female	60,94	37,18	III	C
HP-008	20	Male	No data	No data	No data	No data
HP-009	53	Male	79,95	51,58	III	C
HP-010	47	Male	26	11	IV	C
HP-011	57	Male	65	23	III	C
HP-012	61	Male	No data	No data	III	B
HP-013	58	Male	94,44	12,5	III	C
HP-014	55	Male	87,88	20,68	IV	C
HP-015	67	Male	97,62	28,79	IV	C
HP-016	58	Female	78,7	15	IV	C
HP-017	65	Female	21,11	48,38	III	C
HP-018	61	Female	51,75	67,5	III	B

DBI: Dental biofilm index; BI: Bleeding index .

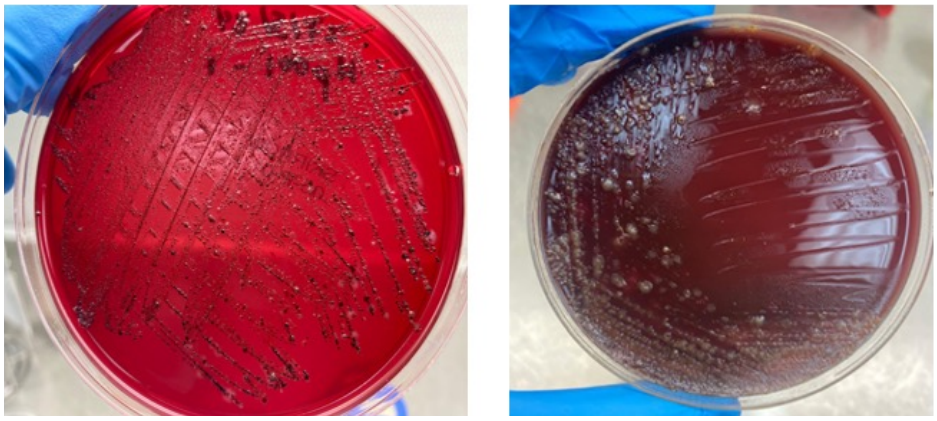

Figure 1. Representative bacterial culture of samples Hp-001 and Hp-015.

Table 2. Demographic and clinical characteristics for H. pylori-positive population (n=17) by stage of periodontal disease.

	Stage II	Stage III	Stage IV
Patients	1	11	5
Sex (F;M)	1	6; 5	1; 4
Age (mean ± st. dev.)	47	55.09 ± 7.92	59.40 ± 9.29
Dental Biofilm Index	76,67	66.94 ± 20.48	71.04 ± 27.90
Bleeding Index	15	35.74 ± 18.28	19.49 ± 6.83
Grading of periodontitis	B: 1	B:2 C:9	B:1 C:4
PCR P. gingivalis			
Positive	0	5	2
Negative	1	6	3

DISCUSSION

This study evaluated the prevalence of *H. pylori* in the SB of patients with periodontal disease. Fecal samples were obtained from 40 participants, with *H. pylori* detected in 55% of samples provided. This high prevalence aligns with existing evidence indicating that over 50% of the global population harbors *H. pylori* (53). Previous studies suggest the oral cavity may serve as an extra gastric reservoir and link *H. pylori* to gastric diseases. The detection of *H. pylori* in SB has also been documented (23-25). Our findings support this evidence, highlighting a high prevalence in patients with periodontal disease and reinforcing the hypothesis of oral colonization. To our knowledge, no previous studies have investigated the presence of *H. pylori* in patients with periodontitis in Costa Rica.

Bacterial culture was performed from SB samples using Skirrow selective medium. This medium contains broad-spectrum antimicrobial agents intended to inhibit Gram-positive cocci (vancomycin) and bacilli, (trimethoprim and polymyxin), thereby selectively promoting the growth of Gram-negative bacilli such as *H. pylori* (26). However, despite the activity of these antibiotics, a wide variety of morphotypes were observed across all cultures, preventing successful isolation of *H.*

pylori, which typically forms small (~1 mm), colorless, smooth, non-hemolytic colonies. This result aligns with reports indicating that the oral cavity harbors a highly diverse microbiota, including microorganisms with varying antibiotic resistance profiles (27).

PCR analysis successfully detected *H. pylori* 16S ribosomal RNA gene in the bacterial DNA extracted from all biofilm samples. However, *H. pylori* DNA was not detected in four samples obtained from bacterial colonies recovered through culturing. This discrepancy may be attributed to the ability of certain microorganisms to enter a viable but non-culturable (VBNC) state in response to environmental stressors that inhibit growth and survival (28). Under adverse conditions, *H. pylori* can transition from its typical bacillary morphology to a VBNC state, characterized by changes in shape, metabolism, and growth patterns. This adaptive response is particularly pronounced within biofilms, where bacterial communities exhibit genomic modifications that enhance resistance, genetic diversity, and environmental adaptability (28). Within the subgingival biofilm, the bacterium adapts by resisting pH fluctuations and coexisting with other bacterial species (29). Given these bacterial adaptations, traditional culture techniques may be insufficient for accurate detection. Consequently, molecular methods are necessary to either supplement or replace cultures.

Eskandari *et al*., in a study of 69 patients, reported a low prevalence of *H. pylori* in dental plaque detected by PCR, with 5.97% positivity in plaque samples and 17.39% in patients diagnosed with gastritis (30). *H. pylori* were mostly absent in patients with periodontitis but without gastritis, indicating an association between *H. pylori* in dental plaque and gastritis (30). Similarly, Liu *et al*. analyzed 443 patients and found *H. pylori* in dental plaque in 59.4% and in the stomach in 61.6%. Gastric infection prevalence was significantly higher in patients with *H. pylori*-positive dental plaque, supporting the link between oral *H. pylori* presence and gastric infection (31). Consistent with these studies, our findings reinforce the evidence that suggest the oral cavity may serve as a reservoir for *H. pylori*, albeit with variable prevalence. This variability reflects the complex relationship between *H. pylori*, the oral environment, and gastric infection. Biofilm samples in this study were obtained from patients with positive stool antigen tests, indicating a potential interaction between gastric and oral reservoirs. Additionally, oral *H. pylori* presence may be influenced by local periodontal conditions and the host immune response (29).

A recent study analyzed the presence of *H. pylori* in patients with gastritis and periodontitis using immunohistochemistry on both gastric and oral mucosa (32). The bacterium was detected in the oral mucosa of both groups. In the stomach, the spiral form predominated, while in the oral cavity the coccoid form -considered VBNC- was more frequent. The authors concluded that the coccoid form should be the primary target for eradication during *H. pylori* treatment (32). In line with this, the four bacterial DNA samples from recovered colonies in our study that lacked detectable *H. pylori* genetic material may have been influenced by metabolic changes. These changes could be attributed to intense competition for nutrients on Skirrow plates due to the high bacterial diversity within the subgingival biofilm, or other environmental factors-including antibiotics and inhibitory substances-that may have induced *H. pylori* into a VBNC state. These findings highlight the importance of molecular methods for detecting *H. pylori* when it exists in the VBNC state (33).

All analyzed samples tested positive for urease activity. As the most abundant enzyme produced by *H. pylori*, urease plays a crucial role in the bacterium's survival under acidic gastric conditions. It catalyzes the hydrolysis of urea into ammonia and carbon dioxide, thereby elevating

the local pH to approximately 4-5. This neutralization of gastric hydrochloric acid creates a more hospitable microenvironment that facilitates the bacterium's migration toward the gastric epithelium. Additionally, ammonia has a chemotactic effect, attracting monocytes and polymorphonuclear leukocytes and stimulating the release of proinflammatory cytokines. This immune response contributes to epithelial inflammation and damage, highlighting urease as a key virulence factor in *H. pylori* pathogenesis (34).

H. pylori is able to adapt and persist in the oral cavity through the production of urease, particularly within periodontal pockets. In this unique microenvironment, urease likely plays a key role by neutralizing acidic metabolic byproducts from other oral microbes and offering protection against fatty acids and antimicrobial agents present in the subgingival biofilm. These adaptations support H. pylori's survival, promote colonization, and facilitate evasion of local immune responses within the biofilm. Just like in the gastric mucosa, urease activity in the oral cavity might also contribute to localized inflammation in the surrounding periodontal tissues (35).

The urease test is a simple and efficient diagnostic tool commonly used to rapidly assess bacterial urease activity in clinical samples. While widely employed for diagnosing *H. pylori* infection in the stomach, its application for detecting *H. pylori* in dental biofilms is limited due to low specificity. This limitation arises from the fact that several other common oral bacterial species-such as *Streptococcus spp.*, *Actinomyces spp.*, and *Haemophilus spp.*-also produce urease, potentially confounding test results (36). In our study, urease positivity alone cannot confirm *H. pylori* presence without molecular confirmation. Notably, previous research (37) reported that bacteria commonly implicated in periodontal disease, including *P. gingivalis*, *Fusobacterium nucleatum*, T*annerella forsythia*, and *Prevotella* intermedia, exhibited no urease activity.

During the analysis of bacterial growth on Skirrow culture media, we predominantly observed small colonies displaying brown or black pigmentation. Given that this colony morphology is commonly associated with Porphyromonas species (38), we proceeded to confirm the presence of *P. gingivalis* through PCR analysis. The analysis for *P. gingivalis* was performed on both DNA extracted from the biofilm samples and DNA from bacterial growths obtained in the Skirrow media. Two biofilm samples couldn't be analyzed due to insufficient material. All other samples, regardless of whether the DNA originated from biofilm (seven samples) or cultures (six samples), tested positive. These results align with evidence identifying *P. gingivalis* as a primary periodontal pathogenic microorganism within the Socransky red complex, alongside *Tannerella forsythia* and *Treponema denticola* (39). Socransky *et al*. suggest that colonization by bacteria not directly linked to periodontal disease can still contribute to the dysbiotic processes of disease development in the oral cavity (40). Consistent with this, observations highlighting the presence of *H. pylori* in the periodontal pocket, identified by molecular methods and microbiome analysis as a member of the oral microbiome, are highly relevant.

In this study, *H. pylori* was detected with greater prevalence compared to *P. gingivalis*, likely because all analyzed samples came from patients with a positive gastrointestinal *H. pylori* result. This aligns with previous studies showing that dental plaque harbors *H. pylori* and that, in positive patients, the oral cavity serves as the primary extragastric reservoir. Furthermore, *P. gingivalis* was found to be more prevalent in individuals with *H. pylori*, suggesting these two bacteria might coexist in the same oral environment (41). This co-relationship within the subgingival pocket can

heighten periodontal inflammation and trigger the release of pro-inflammatory cytokines such as IL-6, IL-8, and TNF-α. These cytokines are crucial in the inflammatory response, promoting tissue destruction and inducing bone resorption, which leads to disease progression (42). Specifically, IL-8 attracts neutrophils to the infection site, exacerbating inflammation and contributing to tissue damage in *H. pylori*-infected patients (42, 43).

An *in vitro* study aimed at evaluating H. pylori's effect on P. gingivalis's pathogenic potential observed changes in the expression of pro-inflammatory cytokines like IL-8 and TNF-α after co-incubation. These observations were attributed to improved expression of P. gingivalis's gingipain RgpB. This model, similar to interactions that can occur in subgingival biofilm, is important for understanding the consequences of the direct interaction between P. gingivalis and H. pylori and how this relationship can accelerate periodontal disease progression (44).

The proposed association between H. pylori presence and periodontal disease progression, through the hypothesis that the bacterium might aggravate periodontal inflammation, aligns with recent theories suggesting H. pylori plays a pro-inflammatory role in periodontal tissues, thereby contributing to disease advancement (45). Due to the limited sample size in this study, we cannot definitively state that H. pylori is increasing the virulence of P. gingivalis in the analyzed biofilms. However, our findings confirm that both bacteria can coexist in subgingival pockets, especially given that positive results for both correspond to patients diagnosed with moderate to severe periodontal disease.

CONCLUSIONS

The oral cavity may serve as a reservoir for *H. pylori*, as its DNA was detected in SB samples by PCR, among the most specific and sensitive methods for detecting *H. pylori* in the oral cavity. While the urease test proved sensitive, its specificity was limited due to the high prevalence of urease-producing bacteria in the oral microbiota. Additionally, isolation of *H. pylori* colonies was unsuccessful, likely due to overgrowth of diverse bacterial morphotypes on Skirrow media, reflecting the complex microbial environment of the oral cavity.

No correlation could be established between *H. pylori* presence and periodontal disease staging and grading, due to the limited number of collected biofilm samples. Further studies are necessary to clarify this potential association. The detection of *H. pylori* in oral biofilm suggests the oral cavity as a potential extragastric reservoir, with implications for preventing gastric reinfection and for comprehensive management of periodontitis. This underscores the need for interdisciplinary collaboration among gastroenterologists, dentists, and other oral health professionals.

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AUTHOR CONTRIBUTION STATEMENT: Conceived the study: M.J.M.S., L.P.H., K.R. and W.M.B.; Obtained funding: K.R.; All authors collected the data; Performed bacterial cultures and urease tests: W.M.B. and M.J.M.S.; Performed molecular characterization: W.M.B.; Analyzed the data: W.M.B.; Interpreted the results: M.J.M.S., W.M.B., V.A.M. and K.R.; Wrote the manuscrip: L.P.H., K.R. and W.M.B.; Reviewed and edited final draft: L.P.H. All authors have read and approved the final manuscript.

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