

# *Helicobacter pylori* Identification: A Diagnostic/Confirmatory Method for Evaluation

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**Abstract** The *Helicobacter pylori* extra gastric reservoir is probably the oral cavity. In order to evaluate the presence of this bacterium in patients with periodontitis and suspicious microbial cultures, saliva was collected from these and non-periodontitis subjects. PCRs targeting 16S rRNA gene and a 860 bp specific region were performed, and digested with the restriction enzyme *DdeI*. We observed that the PCR–RFLP approach augments the accuracy from 26.2 % (16/61), found in the PCR-based results, to 42.6 % (26/61), which is an excellent indicator for the establishment of this low-cost procedure as a diagnostic/confirmatory method for *H. pylori* evaluation.

## Introduction

The bacterium *Helicobacter pylori* has been associated to gastric pathologies and lymphomas [20]. Several routes of bacterial transmission have been postulated, namely oral–oral [2], and strong supporting evidence has led to the identification of oral cavity, including dental plaque and saliva, as a possible extra gastric reservoir of the microorganism [17, 23].

A gold standard method that unequivocally detects *H. pylori* in clinical and environmental samples have not been fully established [1, 3, 5, 13, 19, 27]. Among the non-invasive techniques used to detect *H. pylori* in oral cavity, the urease method is probably the most common choice [3, 27], though the presence of urease producing bacteria in mouth flora may hamper the universal applicability of this methodology [27].

Another useful and clinically non-invasive approach is the polymerase chain reaction (PCR), with the advantage of detecting non viable organisms [23, 27]. However, the inconsistency of detection rates in dental plaque and saliva samples by PCR methods, ranging from 0 to 90 %, has hindering the definition of this technique as a gold standard method [4, 12, 14, 18, 21, 23, 24]. The disparity of detection rates may be ascribable to the presence of PCR inhibitors or the bacteria in the samples are scarce, which are conducive to false negative results. On the other hand, the higher detection rates may be associated with false positive results, due to the presence of non-*H. pylori* bacteria with genome sequence similarities [9, 25]. In an attempt to circumvent these incongruences, target genes with good reports of sensitivity and sensibility have been proposed to detect *H. pylori* in saliva samples [25].

Our group observed mobile and suspicious *H. pylori* bacteria in microbiological cultures established from patients with periodontitis. In order to clarify the presence of this species, we collected saliva samples from those patients and extracted bacterial DNA. After a careful choice of primers, we attempted to establish a simple, sensitive, and specific methodology to evaluate by PCR, the presence of *H. pylori* in saliva samples of subjects with or without periodontal disease by the amplification of three distinct target genes.

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**Table 1** Periodontitis status by gender

	Men	Women	Total
Periodontitis	21	10	31
Non-periodontitis	11	19	30
Total	32	29	61

The median age of the studied population was 40.32 years (SE:  $\pm 2.122$ ; range 20–79 years)

## Materials and Methods

### Patients

Individuals attending the Dental Clinic of the Dentistry School of Instituto Superior de Ciências da Saúde do Norte (ISCSN) were asked to participate in the study, which was previously approved by the local ethics committee. Informed written consents, in accordance with the Helsinki declaration, were obtained from each participant. Table 1 summarizes the studied population according to the presence or absence of periodontitis and gender.

### Microbiological Cultures

Inocula of reference strains from *Helicobacter pylori* (CIP 101260), *Campylobacter sputorum* (CIP 103749), *Arcobacter cryaerophilus* (CIP 103727), and *Aggregatibacter actinomycetemcomitans* (ATCC<sup>®</sup> 33384<sup>TM</sup>) was established.

### DNA Extraction

One milliliter of unstimulated whole saliva was collected into a sterile 1.5 ml microcentrifuge tubes, and stored at  $-80^{\circ}\text{C}$  until molecular analysis was performed. Total DNA was extracted from the saliva using a commercial available kit (QIAamp<sup>®</sup> DNA Mini kit; Qiagen, GmbH, Hilden, Germany) according to the manufacturer's recommendations, with minor alterations. The isolated DNA was eluted in 200  $\mu\text{l}$  of distilled and apyrogenic water. The quality and concentration of the extracted DNA was assessed by means of a Nanodrop Spectrophotometer (ND-2000 Spectrophotometer, Wilmington, USA). DNA samples with spectrophotometer ratios (Abs 260/280) between 1.5 and 2.0 were considered acceptable to study inclusion.

### PCR Analysis

Three sets of primers targeting different genes, namely 16S rRNA region, urease A gene, and a 860 bp DNA specific region, described as sensitive and specific for detection of *H. pylori* in saliva samples [23–25], were chosen. The sequences of primers were previously aligned in a

sequence database using the BLAST program to re-check their sensitivity and specificity for *H. pylori*. The PCRs amplification cycles were performed in a Techne<sup>®</sup> Progene thermocycler (FR, PROGMale5D, Duxford, Cambridge, UK). The sequences and the reaction conditions for each primer set are described in Table 2.

DNA extracted from the standard reference *H. pylori* culture (CIP 101260) was run as the positive control in all PCR experiments. For establishing negative controls, DNA from the standard reference cultures *C. sputorum* (CIP 103749) and *A. actinomycetemcomitans* (ATCC<sup>®</sup> 33384<sup>TM</sup>) were run in each assay. These species present cross-reactivity to *H. pylori* [7, 8] and all may share the same ecological niche [13]. Thus, this methodological approach could rule-out false positive results and add quality control to the PCR diagnostic tests. To control a possible reagent or environmental contamination, non template samples containing all the PCR reagents except template DNA were run in each amplification batch. All PCR reactions were repeated at least twice.

### Analysis of PCR-Amplified Products

Five microliter of the amplified products were analyzed by electrophoresis in 0.8–2 % (w/v) agarose gels (Agarose MB0270; Nzytech; Lisboa, Portugal) in TAE buffer 1 $\times$ , ethidium bromide stained (0.5  $\mu\text{g}/\text{ml}$ ), visualized under ultra-violet light, photographed, and then compared with molecular weight standards.

### Criteria for Labeling the Results as Positive

The detected organism was labeled as *H. pylori* when simultaneous amplification of two or more genes of the bacterium were noticed, negative if none gene was amplified, and inconclusive whenever only one amplicon was observed or the results were inconsistent after repeating three PCR reactions.

### Sensitivity Determination

False negative results may be attributable to the low number of organisms or the inhibitors present in the samples. In order to establish the limits of detection of the PCR primer pairs C97/C98, EHC-U/EHC-L, and HPU-1/HPU-2, from a suspension obtained from an inoculum of *H. pylori* strain (CIP 101260) in 1 ml of water, a serial of ten-fold dilutions were performed until reaching 1,000 CFU/ml. Since using DNA isolated from *H. pylori* standard strains cannot mimic the true environmental conditions of diagnostic samples, the same amount of bacteria inoculum of the serial dilution previously established was diluted in 1 ml of saliva of a *H. pylori* negative donor (from  $10^7$  to

**Table 2** The sequences of primers, reaction conditions and amplicons' characteristics

Gene	Primer sequence	Reaction condition	Amplicon (bp)	Nucleotide <sup>b</sup>
16S rRNA	<sup>a</sup> (Outer) <b>C70</b> : 5' AGA GTT TGA TCC TGG C3'; <b>B37</b> : 5'TCG TAA CAA GGT AAC CGT A 3'	95 °C-5 min [95 °C-45 s, 50 °C-45 s and 72 °C-45 s] 25 cycles; 72 °C-7 min	1467	1207626–1209090 1511182–1512649
	<sup>a</sup> (Inner) <b>C97</b> : 5' GCT ATG ACG GGT ATC C 3'; <b>C98</b> : 5' TGG TGT AGG GGT AAA ATC 3'	95 °C-5 min [95 °C-45 s, 50 °C-45 s, 72 °C-45 s] 35 cycles; 72 °C-7 min	396	1208441–1208837 1512001–1512395
860 bp DNA-specific region	<b>EHC-U</b> : 5' CCC TCA CGC CAT CAG TCC CAA AAA 3'; <b>EHC-L</b> : 5' AAG AAG TCA AAA ACG CCC CAA AAC 3'	95 °C-5 min [95 °C-45 s, 60 °C-45 s; 72 °C-45 s] 35 cycles; 72 °C-7 min	412	80076–80488
ureA	<b>HPU1</b> : 5' GCC AAT GGT AAA TTA GTT 3'; <b>HPU2</b> : 5' GTA AAA ACA ATT AAG GAG 3'	95 °C-5 min [95 °C-30 s, 45 °C-1 min, 72 °C-1 min], 35 cycles; 72 °C-7 min	411	77653–77243

<sup>a</sup> The presence of 16S rRNA target was evaluated by a nested PCR; the outer and inner primers sets are specific for *Helicobacter* genus [23].

<sup>b</sup> The genome sequence chosen for reference was *Helicobacter pylori* 26695 (GenBank: CP003904.1; GI 409893163)

1 CFU/ml). Next, 1 ml of each bacterial suspension was used to extract genomic DNA using the same DNA isolation methodology described above. The DNA obtained was eluted in 200 µl of distilled and apyrogenic water, and amplified with each primer pair.

#### Identification of Possible Co-infection with *Campylobacter* and *Arcobacter* spp

The *Helicobacter*, *Campylobacter*, and *Arcobacter* genera form a phylogenetically distinct group. The lack of standardized phenotypically differential methodologies, turn the molecular identification approaches promising tools. To guarantee the specific detection of *H. pylori*, independently of *Campylobacter* and *Arcobacter* co-infection, RFLP (restriction fragment length polymorphism) analysis was performed. Based on a previous study [7], a 1004 bp 16S rRNA fragment common to *Helicobacter*, *Campylobacter*, and *Arcobacter* strains was amplified using CAH16S 1a and CAH16S 1b primers. The *A. actinomycetemcomitans* strain was also analyzed. Six samples previously studied for *H. pylori* presence (two negative, two positive, and two inconclusive) were elected. DNA isolated from the reference strains were used as control samples, individually and as mixtures, in order to establish the restriction patterns. The standard strains mixtures mimic co-infection cases. Four mixtures were prepared and eluted in H<sub>2</sub>O. The Mix A was composed by DNAs isolated from CIP 101260, CIP 103749, CIP 103727; the Mix B by DNAs isolated from CIP 101260, CIP 103749, CIP 103727, ATCC<sup>®</sup> 33384<sup>TM</sup>; the Mixes C and D by DNAs from CIP 101260, CIP 103749, CIP 103727, and *H. pylori* negative and positive samples, respectively. Amplified products were visualized by 1 % (w/v) agarose gel electrophoresis and stained with ethidium bromide (0.5 µg/ml) in TAE buffer 1×. Ten

microliter of PCR products were digested with 10 U restriction enzyme *DdeI* (New England Biolabs, Ipswich, MA, USA), in a final volume of 20 µl at 37 °C for 3.5 h. The reaction was stopped by chilling the restriction products. Restriction fragments were separated by electrophoresis in 2 % (w/v) agarose gel, in TAE 1× buffer, at 90 V for 1 h and visualized after staining with ethidium bromide and UV transillumination. A 100-bp DNA ladder was used as a standard for molecular size determination. To assess the reproducibility, all samples were analyzed at least two times in distinct batch experiments.

#### Restriction Enzyme Digestion of PCR-Amplified Products

To attempt to establish a cost effective and easy-to-use method to quickly attest the PCR-based results obtained, PCR-amplified products with the primer sets EHC-U/EHC-L and C97/C98 were digested with the restriction enzyme *DdeI* (New England Biolabs) in appropriate buffer solution. Among the restriction enzymes available in our laboratory, the restriction map of *DdeI* was the best to discriminate the restriction fragments for both amplification products. To ensure the sensitivity and specificity of the methodology, digested amplicons from six specimens previously classified (two negative, two positive, and two inconclusive) were analyzed by electrophoresis in 2 % (w/v) agarose gel (Agarose MB0270; Nzytech). The size of digested DNA fragments was earlier estimated from the analysis of restriction sites in the target sequences, and subsequently matched up to the migration distances of molecular weight standards. The digestion patterns were compared with saliva specimens and positive (*H. pylori*—CIP 101260) and negative control samples (*C. sputorum*—CIP 103749; *A. actinomycetemcomitans*—ATCC<sup>®</sup> 33384<sup>TM</sup>). The

**Table 3** PCR amplification results and sample classification for the presence of *H. pylori*

Group	PCR						PCR-RFLP					
	Positive		Negative		Inconclusive		Positive		Negative		Inconclusive	
	M	F	M	F	M	F	M	F	M	F	M	F
Periodontitis	2	1	2	1	17	8	2	1	9	1	10	8
Non-periodontitis	0	3	2	5	9	11	0	4	3	6	8	9
	9.8 %		16.4 %		73.8 %		11.5 %		31.1 %		57.4 %	

reproducibility of the method was ascertained by repeating at least twice all PCR-RFLP reactions. Afterward, all available samples were evaluated by PCR-RFLP for *H. pylori* presence.

### Statistical Analysis

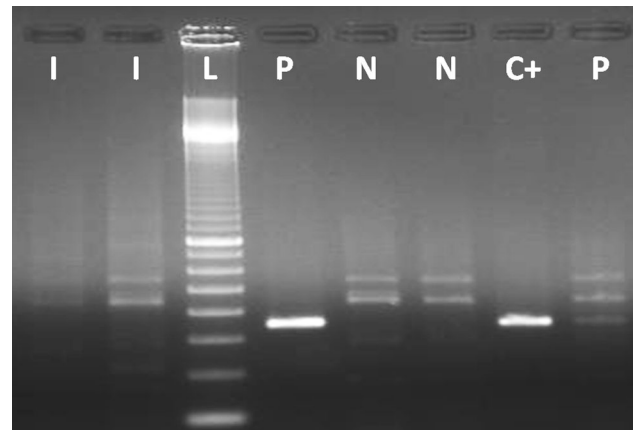
Fisher's or Chi square tests were used to analyze the association between the periodontitis status and the presence of *H. pylori* and to compare the classification of the samples between methodologies. Statistical analysis was performed using the Statistical Packaging for Social Sciences software, version 19 (SPSS Inc., Chicago, IL, USA).

## Results

### Detection of *H. pylori* from Saliva Samples by PCR and by PCR-RFLP

Sixty one samples were analyzed using one nested and two single-step PCRs. Out of these, and in the light of the established classification criteria, six samples were considered *H. pylori* positive (6/61; 9.8 %), ten negative (10/61; 16.4 %), and 45 inconclusive (45/61; 73.8 %) (Table 3). *H. pylori* was detected in four women (one with and three without periodontitis) and in two males belonging to "periodontitis group" (Table 3).

It was ascertained that the rate of *H. pylori* detection was variable between each primer set: by nested PCR (gene target: 16S rRNA; Table 2) the frequency of *H. pylori* positive samples reached 70.5 % (43/61), though by single-step PCR, targeting 860 bp DNA specific region (Table 2), was merely 9.8 % (6/61). All the positive cases discriminated with the last set of primers were also positive for the former. Moreover, the frequency of samples *H. pylori* negative using the Song et al. (2000) [24] method or the nested PCR approach was different (30/61 versus 18/61), and ten samples were classified as negative by both methods. Thus, in line with these results, we opted to use at least two primer pairs targeting distinct genes to accurately evaluate the presence of *H. pylori* in saliva samples.



**Fig. 1** PCR-RFLP digestion patterns with *DdeI*. The 396 bp amplicon obtained for *H. pylori* positive samples with C97/C98 primers after digestion resulted in three restriction fragments [351 bp, 27 bp, and 20 bp (the last two are not visible)]. *P H. pylori* positive sample, *N* negative, *I* inconclusive, *C+* positive control sample (CIP 101260). *L* 100 bp ladder (Invitrogen™, Life Technologies, Carlstad CA, USA)

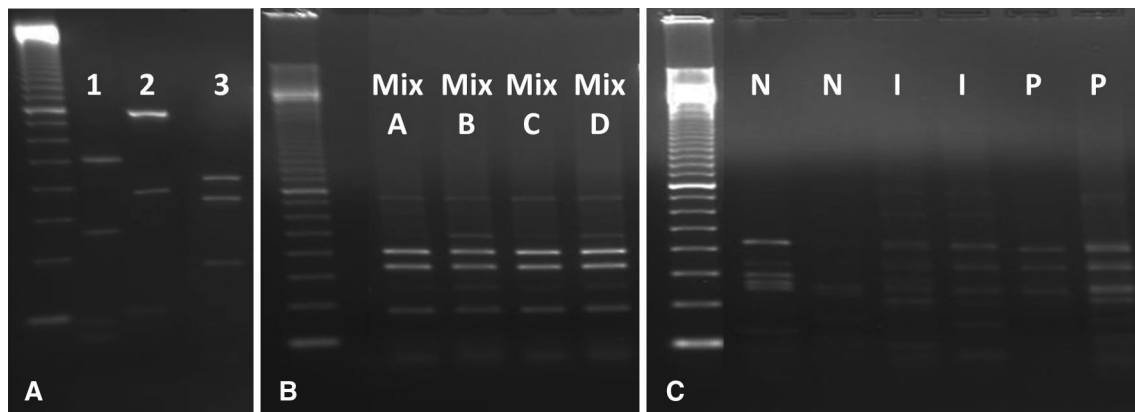
None of the samples was positive for urease A gene, except the positive control sample, derived from a patient with gastritis.

The detection limits of the primer sets found were: HPU-1/HPU-2,  $10^5$  UFC/ml; C97/C98, 1 UFC/ml, and  $10^3$  UFC/ml for the EHC-U/EHC-L.

The PCR-RFLP analysis confirmed the PCR-based classification for all *H. pylori* positive or negative samples (Fig. 1). From the 45 inconclusive samples, one turned out to be classified as positive and nine negative. Due to DNA unavailability, 35 samples (57.4 %) remained to be classified as inconclusive. In summary, we concluded by PCR-RFLP strategy that *H. pylori* was present in 11.5 % (7/61) of the samples and absent in 31.1 % (19/61) (Table 3).

With this methodological approach five women (four belonging to the "non-periodontitis group") and two men with periodontitis were considered *H. pylori* positive.

Nevertheless, the differences between the classification of the samples according to the methodology used, PCR- or PCR-RFLP-based, were not statistically significant ( $P > 0.05$ ). It was also not possible to establish statistically significant association neither with the presence of *H.*



**Fig. 2** PCR–RFLP digestion pattern analysis of amplicons obtained with CAH16S 1a and CAH16S 1b primers. **a** 1 *H. pylori* (CIP 101260), 2 *C. sputorum* (CIP 103749), 3 *A. cryaerophilus* (CIP

103727). **b** Mixs A–D. **c** clinical samples: *P. H. pylori* positive samples, N negative, I Inconclusive. Molecular marker NzyDNA Ladder III (Nzytech)

*pylori* and periodontitis status ( $P > 0.05$ ) nor with gender ( $P > 0.05$ ).

#### Evaluating Co-infection with *Campylobacter* and *Arcobacter* Genera

The analysis to differentiate *Campylobacter*, *Arcobacter*, and *Helicobacter* genera using the method described by Gonzalez et al. [7] reproduced the restriction pattern of each reference strain (Fig. 2a), but is not proper to evaluate clinical samples: mixtures A–D and the positive, negative, and inconclusive saliva samples for *H. pylori* did not present distinct restriction profiles (Fig. 2b, c).

## Discussion

*Helicobacter pylori* infection remains one of the most prevalent chronic bacterial diseases worldwide, affecting more than half of the world population, with a distribution correlated with the degree of economic development [15, 26]. However, fewer than 20 % of the carriers will present symptoms or progress to an overt clinical phenotype associated with the infection [26], which can be explained by the virulence of the strains, as well as with interactions between bacterium/host and/or the environment [15].

Conflicting published results concerning the frequency of *H. pylori* infection are commonly found [6, 15, 16, 22, 26], due to the varying infection rates among populations, and the lack of standard methodological procedures of detection.

Molecular methods, namely PCR based, have become promising tools. However, the interpretation of the results depends upon the biological samples studied, the uniformity of gene sets elected, and the chosen primers [4, 10, 11, 14, 21, 24, 25]. Taking this into account, we tried to establish a PCR–RFLP-based methodology to detect *H.*

*pylori* in saliva samples, which could be easily implemented and economically viable, avoiding the use of sequencing methodologies, and with acceptable rates of sensitivity and specificity.

The analysis of the chosen primer sets individually revealed that the rate of *H. pylori* positive cases by nested PCR was higher than in other published studies [23, 25], which probably reflects false positive results, once after PCR–RFLP analysis this result was not sustained. The rates of detection previously described [23, 25] reached about 30 %, and in our study overtook 70 %, which may indicate that this approach is prone to false positive results.

Regarding EHC-U/L set, the rate of detection was lower than in other studies [4, 25]. However, the rate of specificity, according to the results obtained after the analysis of restriction profile, approximately reach 100 %, which is in accordance with reported outcomes [4, 25].

The urease A gene was undetectable. This reflects probably the extremely reduced level of detection of the chosen primers ( $10^5$  UFC/mL), which obviously would hamper the evaluation. In saliva samples the co-infection with other urease producing species may inhibit by primer-competition the formation of the amplicon [3, 18]. In literature, conflicting results have been published. Medina et al. [16], using the same primer set that we chose, achieved a similar rate of detection (51.6 %) of *H. pylori* in saliva and dental plaque samples of dyspeptic patients. On the other hand, despite using different primer sets, Chaudhry et al. [3], in line with our results, did not obtain any amplicon.

Having all these data into consideration, we suggest that the analysis of several target sequences of *H. pylori* is probably the best method to detect the bacterium in saliva samples.

The present study also highlighted that PCR–RFLP approach augments the accuracy from 26.2 % (16/61),

found in the PCR-based results, to 42.6 % (26/61). In the literature, accuracy is a parameter of quality rarely considered to assess the quality of *H. pylori* detection tests, which blocks the comparison between studies. To the best of our knowledge, this is the first report that compares specifically the accuracy of PCR- versus PCR-RFLP-based methods, and in our opinion it might be a good indicator for future applications as a diagnostic/confirmatory method for *H. pylori* evaluation.

The oral colonization rates according to sex are very variable [6, 15, 16]. In this study, the *H. pylori*-positive samples were predominantly found among women, although the differences between genders were not statistically significant. The prevalence of *H. pylori* among children reaches about 80 % [15, 26], which may be associated with the strong interaction mother-child. The intimate contact of the mothers with their babies and the putative higher susceptibility of young children to become infected with a small load of microorganisms may support the hypotheses that *H. pylori* is transiently present in oral cavity and may have a relevant role in the transmission of the infection. These results can unveil the need of adding screening tests of *H. pylori* contamination of future mothers. In order to test this hypothesis, we wish to increment the representativeness of women in periodontitis and non-periodontitis groups of patients.

In summary, these results indicate that the combination of methodologies could be a useful and accurate tool to clearly identify the presence of *H. pylori* in biological/clinical samples. The cost and time effectiveness are possible advantages to a global implementation of this screening methodology.

In future studies, we must confirm by the same methodological approach all the inconclusive samples, which together with the sequencing of the amplicons and the gathering of saliva samples paired with gastric biopsies and dental plaque, would allow us to accurately establish the sensitivity and the specificity of our results. Additionally, we also have to discard the possibility of false positive or negative results due to co-infections, since we showed that the methodology developed by Gonzalez et al. [7] is not applicable to clinical samples.

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