

Quantitation of *Helicobacter pylori* in dental plaque samples by competitive polymerase chain reaction

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Abstract

Aim—To establish a competitive PCR (cPCR) assay for quantitation of *H pylori* organisms in dental plaque samples.

Methods—The cPCR coamplified target *H pylori* DNA and a known amount of internal standard template in the same tube with the same primers directed to 0.86 kb DNA of *H pylori*. The internal standard was a synthesised DNA bearing the same primer recognition sites at two ends and a non-homologous core sequence as the target DNA fragment. Quantitation was based on determination of the relative, not absolute, amounts of the differently sized and [³²P]-dCTP labelled products derived from *H pylori* DNA and the competitive internal standard after gel electrophoresis separation.

Results—A significant correlation between known amounts of *H pylori* added to dental plaque samples and the results of the cPCR was found, and a standard line was developed which allowed quantitation of *H pylori* in the plaque samples. cPCR was performed on supragingival plaque samples from 10 adult patients with *H pylori* infection in the stomach, and from five adults and six children without *H pylori* infection in the stomach. The ranges of *H pylori* numbers were 1–213 (median 25), 6–76 (10), and 4–94 (14) cells/mg of dental plaque in the three groups, respectively.

Conclusions—cPCR is useful for quantitation of *H pylori* in supragingival dental plaque samples; however, the number of the organisms in dental plaque samples seems very low.

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Keywords: *Helicobacter pylori*; competitive PCR; dental plaque

There is an increasing body of evidence that *H pylori* DNA may often be identified in samples from dental plaque of patients with and without *H pylori* infection in their stomachs.^{1–8} Discrepant results concerning the prevalence of *H pylori* in the oral cavity may be caused by different methods or different study populations. Nevertheless, a quantitative analysis of *H pylori* organisms in oral samples might be useful because of its high sensitivity and specificity compared with other diagnostic methods, which require relatively large numbers of bacteria to obtain positive results.^{9–12}

Several quantitative PCR assay methods have been reported. Competitive PCR (cPCR) is appropriate for the quantitation of small

amounts of the target sequence because there is no need to restrict the number of amplification cycles, and this increases the sensitivity of the assay.¹³ This method has proved useful in quantitating mRNA and DNA of micro-organisms,^{14–18} including *H pylori* in gastric mucus.¹⁹ cPCR coamplifies two different templates bearing the same primer recognition sequences in the same tube. Therefore any variable influencing the amplification reaction should affect both templates similarly, and the relative abundance of coamplified products should remain constant even under different conditions of amplification efficiency. The ratio of PCR products is related to the initial template concentration. Among the two templates, one is a competitive internal standard of known concentration and the other is a target sequence of unknown amount. Therefore the initial quantity of the target sequence can readily be determined from a known amount of internal standard.^{13 20}

Our aim in this study was to establish a cPCR assay for quantitating the numbers of *H pylori* organisms in dental plaque samples.

Methods

SUBJECTS AND SAMPLES

Dental plaque samples from supragingival location used in this study were collected by dentists from symptomatic adult patients who underwent gastroduodenoscopy in the University Clinic of Ulm and from children under orthodontic treatment in the Department of Orthodontics, University of Ulm. Informed consent from the patients or from the parents of the children was obtained in each case. The study was approved by the ethics board of the University of Ulm. Before endoscopy and before incorporation of an orthodontic device, dental plaque samples were scraped from different locations and transferred into tubes containing sterile physiological saline. The curettes used in this study are sterilised and washed thoroughly before autoclaving. It has been shown by Roosendaal *et al* that false positive PCR results can be obtained by contaminated instruments used for collecting samples.²¹

Dental plaque was weighed and stored immediately at –20°C until DNA extraction. Active infection with *H pylori* was determined by the ¹³C-urea breath test (UBT) in each case.²² Patients who had taken drugs such as antibiotics, proton pump inhibitors, H₂ blockers, antacids, and bismuth compounds within the last three months were excluded. These drugs are known to interfere with the urea breath test, leading to a false negative result.

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DNA PREPARATION FOR PCR

In order to avoid false positive results in the nested PCR owing to contamination in the laboratory, great care was taken at all steps in the preparation of the samples; thus we used fresh disposable devices, prepared template DNA, pre-PCR, and post-PCR materials in separate places, changed gloves frequently, and applied other measures outlined by Kwork and Higuchi.²³

DNA extraction from dental plaque samples was as described by Li *et al.*⁴ Briefly, the dental plaque samples were vortexed thoroughly and washed with physiological saline. The pellets were suspended in digestion buffer (0.1 M NaCl / 0.01 M Tris HCl [pH 8.3] / 0.25 M EDTA / 1% sodium lauryl sarcosine) containing proteinase K (final concentration 100 µg/ml), and incubated at 55°C for three hours. DNA was extracted with phenol-chloroform, precipitated with isopropanol, and washed with 70% ethanol. Each DNA pellet was resuspended in 150 µl of sterile water. The DNA samples were then investigated for the presence of *H pylori* DNA by nested PCR using primers EHC-U/EHC-L (5'-CCCTCACGCCATCA GTCCCAAAA3'/5'-AAGAAGTCAAAAAC GCCCAAAAC-3')⁴ and ET-5U/ET-5L (5'-GGCAAATCATAAGTCCG CAGAA-3'/5'-T GAGACTTTCCTAGAAGCGGTGTT-3'); courtesy of Li C, East Tennessee State University, USA), which were directed to an 860 base pair (bp) fragment of *H pylori* DNA. The region targeted by the primers EHC-U/EHC-L is located in 80076-80492 bp of the genome of *H pylori*.²⁴

The primer set ET-5U/ET-5L, which is internal to the fragment amplified by EHC-U/EHC-L, had an expected product size of 230 bp (corresponding nucleotides, 446 to 675 of the 860 bp DNA of *H pylori*; Thomas E, Li C, East Tennessee State University, Johnson city USA; personal communication).

The samples that were *H pylori* DNA positive by the nested PCR were further investigated by quantitative cPCR.

COMPETITIVE PCR

cPCR was performed as described by Gilliland *et al* and Sambrook *et al.*^{13, 20} The primer set used was EHC-U/EHC-L. The competitive internal standard was a synthesised single strand DNA (140 bases) containing the same primer recognition sites at the two ends, and a non-homologous core sequence as the target 417 bp fragment. The internal standard and the target 417 bp fragment have similar G + C compositions (38.6% and 36.2%, respectively) based on the sequence of the 417 bp fragment (Thomas E, East Tennessee State University, USA; personal communication). Precise 10-fold serial dilutions of internal standard, ranging from 10⁰ to 10⁻⁹ pg/µl, were prepared in a relatively large volume (1 ml) so that the same dilution series could be used for multiple concentration determinations. For PCR amplification, six identical portions of DNA suspension of each sample were added into six parallel reactions, each of which contained decreasing known amounts of internal standard. Each

reaction was performed in a 25 µl volume, containing 2.5 µl of 10 × PCR buffer (100 mM Tris HCl [pH 9.2] / 15 mM MgCl₂ / 750 mM KCl), 0.5 µl of 10 mM dNTPs (final concentration 0.2 M), 25 pM of each primer, 5 µl of dental plaque DNA, 2.5 µl of internal standard DNA, 1.25 U of Taq polymerase, and 0.125 µl of ³²P-α-dCTP (final concentration, 50 µCi/ml). Each tube was overlaid with 25 µl of mineral oil. The amplification consisted of the initial denaturation at 95°C for five minutes, 40 cycles with denaturation at 94°C for 45 seconds, annealing at 59°C for 30 seconds, extension at 72°C for 45 seconds, and the last extension at 72°C for 10 minutes. A negative control (without target DNA and internal standard) was used to monitor false positivity and to determine the background of radioactivity.

After amplification, 10 µl aliquots of PCR products were analysed by electrophoresis on 2% agarose gels containing ethidium bromide. The 417 bp bands (*H pylori* DNA) and the 140 bp bands (internal standard DNA) were excised from the gels and the amounts of radioactivity counted in a scintillation counter. The radioactivity of the 140 bp band was multiplied by ratio of G + C in the 417 bp fragment to that in the 140 bp fragment to correct for increased label per mol by the larger fragment. The point of equivalence, where yield of target fragment equalled that of internal standard, reflected equal starting amounts of target *H pylori* DNA and internal standard DNA (fig 1).

DEVELOPMENT OF STANDARD LINE

First, a suspension of cultivated clinical isolates of *H pylori* was prepared and the number of *H pylori* cells determined with a Thoma chamber. Aliquots were diluted to concentrations of 10⁶, 10⁵, 10⁴, 10³, 10², 10¹, and 10⁰ *H pylori* cells/µl with *H pylori* negative dental plaque suspension.

Dental plaque samples with negative PCR result for *H pylori* DNA were only detected in patients who had recently been treated with anti-*H pylori* triple therapy, or who had received antibiotic treatment for other reasons. The pooled dental plaque from these subjects was used for the dilution experiments and for the construction of the standard line.

DNA was then prepared from these mixtures using the method described above. Finally, the quantity of *H pylori* in each mixture was determined at least twice by the cPCR method. The correlation coefficient *r* was calculated for the association between the actual quantity of *H pylori* in the suspensions and the results of the cPCR assay. The statistical significance of the *r* value was assessed, and the regression line and equation were established. This relation was used as the standard for quantitation of *H pylori* in the dental plaque samples.

Results

Ten *H pylori* positive adult patients (six women, four men; mean age 49.1 years), five *H pylori* negative adult patients (one woman, four men; mean age 46.8 years), and six *H pylori* negative children (four girls, two boys; mean age 11 years) were selected to evaluate

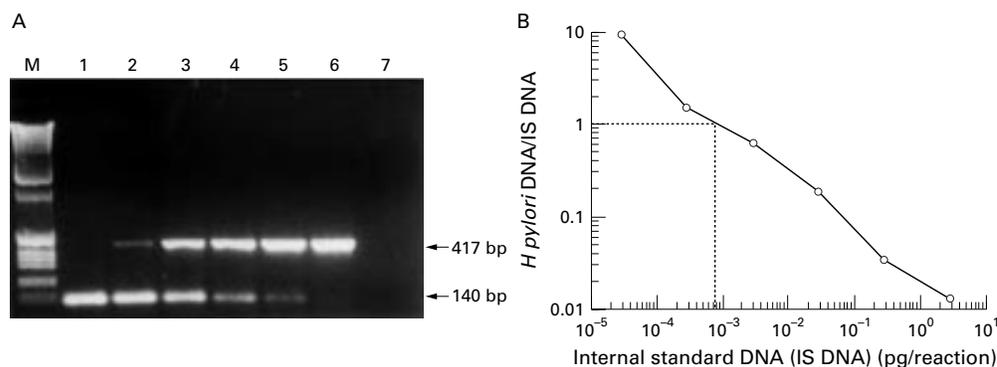


Figure 1 Competitive polymerase chain reaction (cPCR) quantitation of *H pylori*. (A) Each PCR reaction contained primers EHC-U/EHC-L, radiolabelled dCTP, a different amount of internal standard DNA (IS DNA), and a fixed amount of DNA extracted from a mixture of *H pylori* and dental plaque. Lane M, 1 kb DNA ladder; lanes 1 to 6, 2.5, 2.5 × 10⁻¹, 2.5 × 10⁻², 2.5 × 10⁻³, 2.5 × 10⁻⁴, and 2.5 × 10⁻⁵ pg of the internal standard DNA, respectively; lane 7, negative control. (B) Data were plotted as log of the ratio of *H pylori* DNA/internal standard DNA v log internal standard DNA. The point of equivalence (ratio = 1) reflects equal starting amount of *H pylori* DNA and internal standard DNA.

the quantitative cPCR method. The demographic data of the patients are shown in table 1. The mean (SD) amount of plaque sample analysed per subject in *H pylori* positive adults was 22.2 (10.8) mg, in *H pylori* negative adults 20.4 (6.7) mg, and in *H pylori* negative children 12.8 (5.3) mg.

STANDARD LINE AND REGRESSION EQUATION

The standard line is shown in fig 2. A significant correlation ($r = 0.998$, $p < 0.001$) was found between the known amounts of *H pylori* and the results of the cPCR assay. The relation was expressed as a regression equation: $Y = 3 \times 10^{-9}X$. Y is the result of the cPCR assay (pg), and X is the amount of *H pylori* DNA (number of organisms) in each PCR reaction.

NUMBER OF *H PYLORI* ORGANISMS IN DENTAL PLAQUE SAMPLES

Table 1 shows the number of *H pylori* organisms per mg of dental plaque sample. Adult patients with active *H pylori* infection showed a

Table 1 Demographic data of patients ($n = 21$) and results of quantitative cPCR

| Patient | Age (years) | Sex | Diagnosis | <i>H pylori</i> status in stomach* | <i>H pylori</i> cell No/mg dental plaque |
|---------|-------------|-----|--------------------|------------------------------------|--|
| 1 | 23 | F | G†, DU‡ | + | 171 |
| 2 | 32 | F | G | + | 11 |
| 3 | 48 | F | G, Crohn's disease | + | 25 |
| 4 | 55 | M | G | + | 53 |
| 5 | 60 | F | G, hiatus hernia | + | 1 |
| 6 | 70 | M | G | + | 25 |
| 7 | 42 | M | G, DU | + | 3 |
| 8 | 58 | F | G, hiatus hernia | + | 30 |
| 9 | 70 | F | G | + | 3 |
| 10 | 33 | M | G | + | 213 |
| 11 | 26 | M | Oesophagitis | - | 6 |
| 12 | 49 | M | Oesophagitis | - | 76 |
| 13 | 64 | M | N§ | - | 16 |
| 14 | 57 | F | N | - | 10 |
| 15 | 38 | M | Hiatus hernia | - | 7 |
| 16 | 12 | M | ¶ | - | 16 |
| 17 | 11 | F | \ | - | 4 |
| 18 | 10 | F | \ | - | 10 |
| 19 | 13 | F | \ | - | 15 |
| 20 | 10 | F | \ | - | 94 |
| 21 | 10 | M | \ | - | 12 |

*Result of the ¹³C-urea breath test; †gastritis; ‡duodenal ulcer; §normal; ¶not done.

range from 1 to 213 *H pylori* organisms (median 25), adult patients without *H pylori* infection showed a range from 6 to 76 *H pylori* organisms (median 10). In children without *H pylori* infection, *H pylori* amount varied from 4 to 94 organisms (median 14). In most samples (79%), less than 50 *H pylori* organisms were found in 1 mg of dental plaque.

Discussion

To our knowledge, this is the first report to quantify *H pylori* in supragingival dental plaque samples, and the results show very few *H pylori* organisms. The small number of *H pylori* organisms, the presence of coccoid forms of *H pylori* (probably caused by the increased oxygen environment of supragingival plaques), and the presence of numerous other microorganisms may have contributed to the failure to cultivate the bacterium from the oral cavity in previous studies.⁹⁻¹² Such low numbers of *H pylori* in dental plaque may also be responsible for the reported variation of PCR detection of *H pylori* in oral samples.^{1 3 10 11 25 26} The PCR detection limit of *H pylori* is usually about 5–100 cells.^{4 26-30} The number of *H pylori* in 1 mg of dental plaque is similar using the PCR detection limit; thus a small change of the

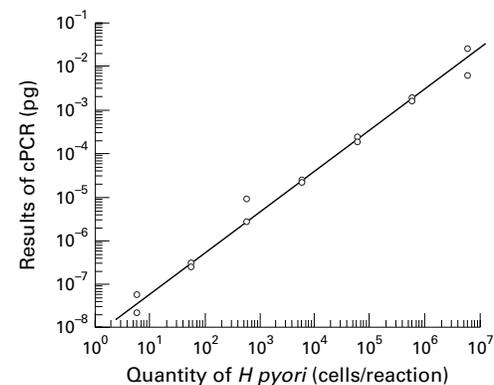


Figure 2 Results of competitive polymerase chain reaction (cPCR) quantitation of a known amount of *H pylori* added to dental plaque. These results of this assay correlate significantly with the actual *H pylori* amounts ($r = 0.998$, $p < 0.001$). The regression line is expressed as: $Y = 3 \times 10^{-9}X$ (Y = result of cPCR assay; X = number of *H pylori*).

detection limit will lead to very different results. However, the primers EHC-U/EHC-L and the 860 bp fragment were shown to be very specific and sensitive for *H pylori* DNA by Li *et al.*,^{4, 30} and also by us in a previous report.³¹ Furthermore, we were able to show a high degree of homology, with more than 97% nucleotide identity when comparing the sequence of the 230 bp products of *H pylori* type strain ATCC 43629 with 39 oral samples from 20 patients (Song Q, *et al.*, unpublished data).

Using a synthesised single strand DNA as internal standard, we found a significant correlation between the known amount of *H pylori* added to dental plaque and the result given by cPCR. Furuta *et al.* obtained similar results using a synthesised single strand DNA as internal standard.¹⁹ Their results of quantitating *H pylori* in gastric mucus were significantly correlated with those of other methods. Therefore, synthesised DNA can be used as an internal standard in cPCR quantitation of *H pylori*. On the basis of our experience, the following conditions are necessary to overcome problems during quantitation of *H pylori* in dental plaque samples with cPCR:

- the use of highly specific primers for *H pylori* to avoid amplification of non-specific bands owing to the presence of numerous DNAs other than *H pylori* DNA;
- the use of a competitive template that is big enough not to overlap with primer dimers;
- thorough vortexing of sample suspensions before DNA preparation to make sure that *H pylori* cells are distributed uniformly and so ensure that results are consistent and reproducible;
- a relatively large volume of specimen DNA should be taken for each PCR reaction.

Nevertheless, results are still less consistent and reliable if the numbers of *H pylori* are below 10 cells per PCR reaction, although positive signals could be detected with as few as 1–5 cells. This is in agreement with previous reports which showed that quantitation was less reliable when the target sequence was extremely small.^{17–32} Quantitative cPCR is very time consuming and is not suitable for studying large samples. Therefore a generalised application of our results is not envisaged.

The method we describe seems extremely sensitive and specific and thus great care has to be taken to avoid sample contamination. We cannot completely exclude the possibility of low level contamination. However, the presence of *H pylori* organisms in dental plaque samples seems to be independent of the presence of the organism in the stomach.

CONCLUSIONS

cPCR is useful for the quantitation of *H pylori* in dental plaque. The number of the bacteria is very small and seems to be independent of infection in the stomach. *H pylori* may belong to the normal flora of the human oral cavity, maintaining a commensal relation with the host. The mere presence of the pathogen in the oral cavity does not appear to mean that an individual will develop the infection in the

stomach. The number of *H pylori* organisms necessary to induce infection and disease in the stomach is still unknown, and further studies are needed to investigate whether the presence of *H pylori* in the mouth is transient and whether there are risk factors that favour its growth in the oral cavity.

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