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Quantitative Study of *Helicobacter pylori* in Gastric Mucus by Competitive PCR Using Synthetic DNA Fragments

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***Helicobacter pylori* is closely related to upper gastrointestinal diseases, and the precise evaluation of *H. pylori* infection is necessary for the treatment of these diseases. The aim of the present study was to establish a method for the quantitative detection of *H. pylori*. We applied a competitive PCR method using various amounts of synthetic DNA fragments containing the same primer-binding and a subset of the same template sequences as the target competing for primer binding and amplification in order to quantify *H. pylori* in gastric mucus. The results obtained by this method were compared with the results of histological examination, the rapid urease test, bacterial culture, the [^{13}C]urea breath test, and urea and ammonia measurements in gastric juice. As the quantity of *H. pylori* in gastric mucus increased, the rates of positivity of histological examination, the rapid urease test, and bacterial culture increased. The quantity of *H. pylori* in gastric mucus was also significantly correlated with the results of the [^{13}C]urea breath test and was negatively correlated with the urea/ammonia ratio in gastric juice. The competitive PCR method provides an objective measure of the quantity of *H. pylori* and makes it possible to distinguish true negatives from false negatives due to incomplete PCR and true positives from false positives due to contamination. This method is very useful for the precise evaluation of gastric *H. pylori* infection.**

A close relationship is now thought to exist between *Helicobacter pylori* and peptic ulcer disease, gastritis, and certain other gastroduodenal diseases (1, 2, 10, 12, 21, 26, 31, 33). The diagnosis of *H. pylori* infection is therefore of great significance in the treatment of these diseases. Recently, a variety of diagnostic methods have been used clinically. Most of them, for example, bacterial culture (7, 11), urease tests (15, 23, 24, 27), and histological examination (22, 41), require the collection of gastric samples by mucosal biopsy during gastroduodenoscopy. Urea breath tests (6, 13, 25, 32, 38, 40) and tests for serum antibodies (17, 20, 30, 42) provide indirect evidence of infection. No entirely satisfactory method has been developed with regard to sensitivity, specificity, and cost. Recently, the nucleotide sequences of segments of the *H. pylori* genome have been determined, and the PCR method has been used for the detection of *H. pylori* in gastric tissue (3–5, 14, 18, 37, 39). The level of sensitivity of this method is high, but it cannot be used to evaluate the quantity of *H. pylori* in clinical samples.

It has been reported that *H. pylori* is present not only on the gastric mucosal cell surface but also in gastric mucus (16). We previously reported a new method for the quantitative detection of *H. pylori* in gastric mucus, competitive PCR (cPCR) with synthetic DNA (sDNA) fragments (9), which was different from the method of Negishi et al. (28). In the present study, we modified this method and compared the results obtained by cPCR with the results of histological examination, bacterial culture, the rapid urease test (RUT), the [^{13}C]urea breath test (^{13}C-UBT), and testing of the urea/ammonia (U/A) ratio in gastric juice.

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MATERIALS AND METHODS

Subjects. A total of 190 patients (114 men and 76 women; age range, 26 to 78 years; mean age, 46.5 years) with endoscopically proven gastric ulcer (n = 106), duodenal ulcer (n = 79), or chronic gastritis (n = 5) were examined, and 46 patients also underwent the ^{13}C-UBT.

In our study group, 113 patients had never received any anti-*H. pylori* treatment. The other 77 patients had received anti-*H. pylori* treatment, but none had received any anti-*H. pylori* treatment for 1 month before the study. All patients had normal renal function. Written or oral informed consent was obtained from all patients.

Collection of clinical samples. Gastroduodenoscopy was performed after a venous blood sample was obtained for determination of the serum urea concentration to exclude azotemia. During gastroduodenoscopy, immediately after entering the stomach, 10 ml of gastric juice was aspirated through the suction channel of the endoscope and was collected in a trap placed in the suction line. Routine inspection of the upper gastrointestinal tract was performed, and then several biopsy specimens from both the antrum and the corpus were obtained for RUT, bacteriological culture, and histology. The endoscopists were blinded to the treatment received by the patient.

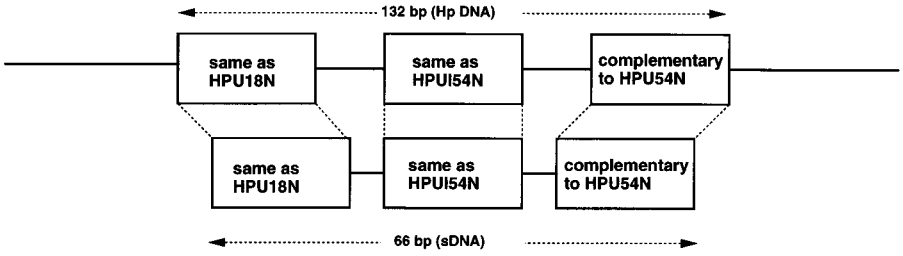
RUT. For RUT, biopsy specimens were inoculated into the STAT UREASE TEST (PML Microbiologicals, Tualatin, Oreg.), and any color change was noted after 30 min and after 24 h. A positive result was recorded when the color changed from yellow to pink within 24 h.

Culture. For bacterial culture, the biopsy samples were inoculated onto agar plates (Dent's medium [7]) containing a special peptone (23 mg/ml), starch (1 mg/ml), sodium chloride (5 mg/ml), horse blood (5%; vol/vol), vancomycin (10.0 mg/ml), trimethoprim lactate (5.0 mg/ml), cefsulodin (5.0 mg/ml), and amphotericin B (5.0 mg/ml) and were incubated at 37°C under microaerobic conditions for up to 7 days. Colonies were identified as *H. pylori* on the basis of morphology in Gram stains and by oxidase and catalase test results and RUT results.

Histological examination. Each fragment in hematoxylin-eosin- and Giemsa-stained sections was carefully screened for the presence of spiral *H. pylori*-like organisms.

^{13}C-UBT. Thirty minutes after endoscopy, 150 ml of 0.1 N citric acid was administered to the patient to inhibit gastric emptying. One minute later, all patients received 20 ml of 0.1 N citric acid containing 100 mg of [^{13}C]urea (99% ^{13}C; EURISOTOP; Commissariat a L'Energie Atomique, Saint Aubin, France) and then 30 ml of 0.1 N citric acid. Before and after administration, patients rinsed their mouths with water. Breath samples were obtained before and 10 min after ingestion of [^{13}C]urea. The breath sample was collected in an air sample bag. The $^{13}CO_2/^{12}CO_2$ ratio (δ value) was measured with a gas isotope ratio mass spectrometer. All results of ^{13}C-UBT were calculated as delta per mil change over the baseline before the administration of [^{13}C]urea.

Measurement of ammonia and urea concentrations in gastric juice. Soon after



HPU18N: 5'-CCCATTTGACTCAATGCGATG-3'

HPU54N: 5'-TGGGATTAGCGAGTATGTCGG-3'

HPUI54N: 5'-CGCCTTTGATGATCATGTTG-3'

sDNA: 5'-CCCATTTGACTCAATGCGATGCCCGCCTTTGATGATCA TGTTGGGCCGACATACTCGCTAATCCCA-3' (Bio-Synthesis,Inc. Texas)

FIG. 1. Oligonucleotide sequences of primers (HPU18N and HPU54N), hybridization probe (HPUI54N), and sDNA used as a competitor. The sDNA contains the same sequence as HPUI54N in the midportion. HPU18N and HPU54N amplify a 132-bp portion of genomic DNA of *H. pylori* (Hp DNA) and sDNA. PCR products are distinguished by size (*H. pylori* DNA, 132 bp; sDNA, 66 bp).

collection the gastric juice samples were centrifuged at 3,000 × *g* and 4°C for 10 min in order to separate the mucus. The ammonia concentrations in the supernatant were measured after dilution in 0.2 M phosphate buffer at pH 7.4 by an enzymatic method (Wako Junyaku Co., Osaka, Japan). Urea concentrations were measured by the diacetylmonoxime method (Wako Junyaku Co., Osaka, Japan), and the U/A ratio was calculated (29).

Extraction of bacterial genomic DNA from gastric mucus. Bacterial genomic DNA was extracted from a 100-μl portion of the gastric mucus precipitated from the centrifuged gastric juice. We used IsoQuick (Micro Probe Co., Garden Grove, Calif.) for DNA extraction, using a modified method (34). The resulting DNA pellet was dissolved in 400 μl of Tris-EDTA buffer (TE; 10 mM Tris hydrochloride and 1 mM EDTA [pH 8.0]).

Development of a cPCR assay for quantification of *H. pylori* in gastric mucus. The primer pair HPU18 and HPU54 designed by Clayton et al. (4) amplifies the 132-bp band. However, the annealing temperature in the PCR is 45°C, which is so low that the nonspecific bands are often amplified. In order to increase the annealing temperature to 55°C, both HPU18 and HPU54 were elongated to 21 bp. These elongated primers were named HPU18N and HPU54N, respectively (Fig. 1). These new primers contain the same sequences as HPU18 and HPU54, respectively, so each of these new primers retains the same specificity as the original primers.

For cPCR, we prepared a competitive template (sDNA) containing the same primer-binding and a subset of the same template sequences as the target competing for primer binding and amplification (Bio-Synthesis, Inc., Lewisville, Tex.). It contains the same sequence as the Southern blotting probe in the midportion, which is named HPUI54N; the sequence contains a sequence complementary to HPUI54 designed by Clayton et al. (4), as illustrated in Fig. 1. The PCR products are distinguished by size (*H. pylori* DNA, 132 bp; sDNA, 66 bp). Precise nine fivefold serial dilutions ranging from 1.0 to 1.0×5^{-8} pg/μl of template were prepared in relatively large volumes (e.g., 20 ml) so that the same dilution series could be used to measure many samples under identical conditions.

We prepared a master mix containing, in a final volume of 46 μl, oligonucleotide primers (HPU18N and HPU54N; 50 mM each as the final concentration), deoxynucleoside triphosphates (200 μM each as the final concentration), 5 μl of 10× PCR buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl, 15 mM $MgCl_2$, 0.1% gelatin), 1.25 U of *Taq* polymerase, and 5 μl of extracted DNA solution. An aliquot of 46 μl of this mixture was added to 4 μl of a previously prepared competitive template of known concentration in a dilution series. Each reaction mixture was overlaid with 100 μl of mineral oil to prevent evaporation. PCR was performed with an automatic thermal cycler (DNA Thermal Cycler PJ2000; Perkin-Elmer, Norwalk, Conn.). The amplification consisted of an initial denaturation of target DNA at 95°C for 5 min and then denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, and extension at 72°C for 1 min. The final cycle included extension for 8 min at 72°C to ensure full extension of the product. The samples were amplified through 35 consecutive cycles.

The completed reactions were analyzed by electrophoresis of a 10-μl aliquot through 3.0% (wt/vol) agarose gels containing 0.5 μg of ethidium bromide per ml. The bands were visualized by excitation with UV light, and 132-bp bands (*H. pylori* DNA) and 66-bp bands (sDNA) were detected (Fig. 2). The identities of the amplicon bands were verified by Southern blot hybridization (36). The gel was photographed, the intensity of ethidium bromide luminescence was measured with a CCD image sensor (Densitograph AE-6900-F, Atto, Tokyo, Japan), and the ratio of 132-bp bands (*H. pylori* DNA)/66-bp bands (sDNA) was plotted for each dose of sDNA added to the reaction tube. The points of equivalence (i.e., where there was a 1:1 ratio) were when the amount of *H. pylori* DNA equaled the amount of sDNA and represented the concentration of *H. pylori* DNA in the unknown (Fig. 3).

In Figure 3, the point of equivalence was at $4 \times 5^{-2.1}$ pg per tube (1.4×10^{-13} g per tube), which reflected the quantity of *H. pylori* in a 100-μl portion of mucus in this case.

When sDNA bands (66 bp) were detectable and the intensities of *H. pylori* DNA bands (132 bp) were zero or lower than that of sDNA in the ninth lane, PCR amplification was performed again without sDNA or with lower doses of sDNA (4×5^{-9} to 4×5^{-10} pg per tube) and with 40 cycles of amplification. When the intensity of the 132-bp band was stronger than that of the sDNA in the first lane, cPCR was performed again with higher doses of sDNA (4×5^{1} to 4×5^{2} pg per tube) and with fewer cycles of amplification (25 to 30 cycles). When neither sDNA bands nor *H. pylori* DNA bands were detectable, the PCR was thought to be obstructed by a specific inhibitor which might be included in the *H. pylori* DNA solution. PCR amplification was therefore performed again with a lower dose of *H. pylori* DNA solution in the master mix.

Development of standard line. To quantify the number of organisms in the

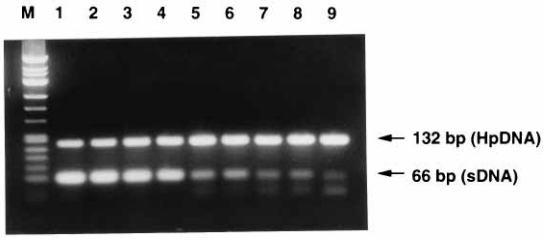


FIG. 2. *H. pylori* DNA (HpDNA; 132 bp) versus sDNA (66 bp). Lane M, size marker (ϕX174 digested with *Hin*fI); lanes 1 to 9, ninefold serial amounts of sDNA (competitor DNA) ranging from 4×5^{0} to 4×5^{-8} pg reacted with a fixed dose of *H. pylori* DNA.

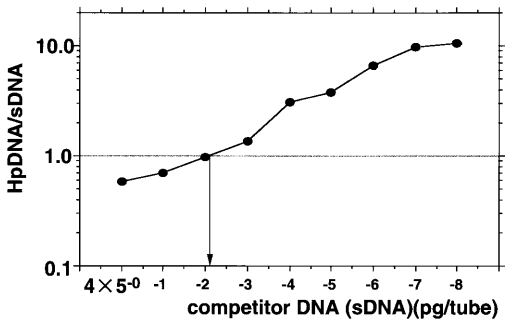


FIG. 3. Logarithmic plot of the ratio of *H. pylori* DNA (HpDNA; 132 bp) to sDNA (66 bp). The quantity of *H. pylori* DNA in the reaction tube was determined by calculating how much of the competitor was required to achieve equal amounts of products. Then, the amount of *H. pylori* DNA was calculated by extrapolating from the intersection of the curves, where the amounts of *H. pylori* DNA and competitor (sDNA) were equal to the *x* axis.

sample, the standard line was made as follows. We first prepared a suspension of clinically isolated *H. pylori*. The concentration was determined by optical density measurement (optical density at 600 nm of 1.0 = 1.6×10^9 organisms per ml) (35) and was confirmed by matching the turbidity to that of a McFarland standard. Aliquots were diluted to concentrations of 1.0×10^8, 1.0×10^7, 1.0×10^6, 1.0×10^5, 1.0×10^4, 1.0×10^3, 1.0×10^2, and 0 organisms per 100 μl with gastric mucus from an *H. pylori*-negative healthy volunteer. The quantity of *H. pylori* in each diluted sample was measured twice by the method described above in order to develop a standard line.

A significant relationship ($r = 0.99$; $P < 0.0001$) was found between the known concentrations of *H. pylori* and the results of the cPCR assay (Fig. 4). The regression line was expressed as follows: $y = 5.6 \times 10^{-9} \times x$. This equation can also be expressed as $x = 1.8 \times 10^8 \times y$ (where *y* is the result of the cPCR assay [in picograms] and *x* is the number of organisms in a 100-μl portion of mucus).

This relationship was used as the standard of quantification. The quantity of *H. pylori* in a 100-μl portion of gastric mucus in the case of Fig. 3, for example, corresponded to $1.8 \times 10^8 \times 4 \times 5^{-2.1} = 2.5 \times 10^7$ organisms per 100 μl.

Data analysis. Pearson's correlation coefficient was computed for the association between the quantity of *H. pylori* in gastric mucus and the results of the cPCR assay, and the statistical significance of the *r* value was assessed by Student's *t* test. The χ^2 test was used to determine the relationship between the quantity of *H. pylori* in gastric mucus and the positivity of culture, RUT, and histology. Spearman's rank correlation coefficient was computed for the association between the quantity of *H. pylori* in gastric mucus and the U/A ratio in gastric juice or the results of the ^{13}C-UBT, and the statistical significance of the ρ value was assessed by Student's *t* test. Findings of $P < 0.05$ were taken to indicate statistical significance. Statistical calculations were performed by using StatView, version 4.5J (HULINKS, Tokyo, Japan) on a Power Macintosh 8100/80AV (Apple Computer, Inc., Cupertino, Calif.).

RESULTS

Success or failure of cPCR amplification. In five samples, neither 66-bp bands nor 133-bp bands were detected. In such samples, cPCR was performed again with a reduced dose of *H. pylori* DNA solution in the master mix (1/5 to 1/10). After the cPCR procedure described above was performed again, 66-bp bands were visualized in all samples.

Relationship between number of *H. pylori* in gastric mucus and results of RUT, bacterial culture, and histological examination. As the quantity of *H. pylori* organisms in the gastric mucus increased, the rate of positivity of RUT, bacterial culture, and histological examination also increased. Our findings indicate that when the number of *H. pylori* is less than 10^5 organisms per 100 μl, most of the results obtained for these tests will be negative (Fig. 5).

Relationship between number of *H. pylori* in gastric mucus and the U/A ratio in gastric juice. Figure 6 describes the relationship between the quantity of *H. pylori* in gastric mucus and the U/A ratio in gastric juice. As the quantity of *H. pylori* in gastric mucus increased, the U/A ratio in gastric juice decreased significantly. When the U/A ratio was greater than 3.0, *H. pylori* was detected in the gastric mucus of a few patients.

Relationship between the number of *H. pylori* in gastric mucus and results of ^{13}C-UBT. Figure 7 describes the relationship between the quantity of *H. pylori* in gastric mucus and the results of the ^{13}C-UBT. As the number of *H. pylori* in gastric mucus increased, the delta per mil change in the $^{13}CO_2/^{12}CO_2$ ratio significantly increased. When the delta per mil change in the $^{13}CO_2/^{12}CO_2$ ratio was greater than 10.0, the quantity of *H. pylori* in gastric mucus was more than 10^6 organisms per 100 μl.

DISCUSSION

A rapid, sensitive, specific, and quantitative test for *H. pylori* would be of great value since there is now evidence of a strong association between *H. pylori* and certain gastroduodenal diseases. One of the goals of the present study was to develop a simple method for the quantitative evaluation of *H. pylori* infection in the stomach.

The conventional standard for the detection of *H. pylori* is bacterial culture. However, its sensitivity is limited because of technical difficulties, and when the quantity of *H. pylori* present is small, positive findings are difficult to obtain. The sensitivity of the RUT is also limited. Both of these methods require biopsy samples, but not all biopsy samples contain a sufficient

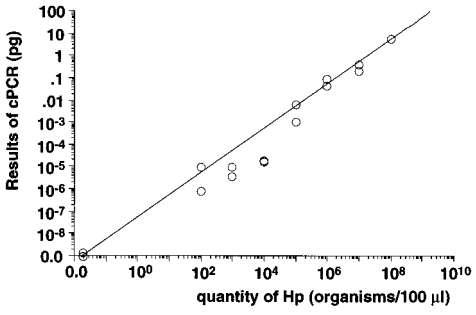


FIG. 4. Results of quantification of a known number of clinically isolated *H. pylori* (Hp) by the cPCR method. The results of this method (in picograms) are significantly correlated with the actual number (organisms per 100 μl) ($r = 0.99$; $P < 0.0001$). The regression line was expressed as follows: $y = 5.6 \times 10^{-9} \times x$ (y, results of cPCR assay [in picograms]; *x*, number of organisms in a 100-μl portion of mucus.)

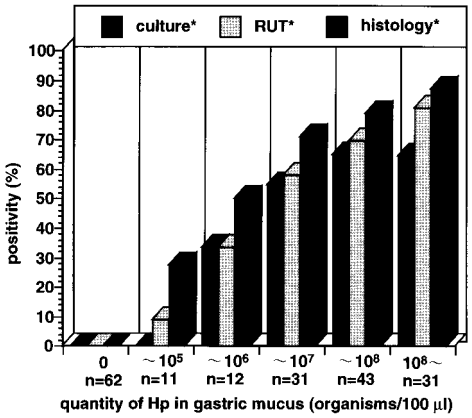


FIG. 5. Relationship between the quantity of *H. pylori* (Hp) in gastric mucus and the results of bacterial culture, RUT, and histological examination. As the quantity of *H. pylori* in gastric mucus increases, positive findings of bacterial culture, RUT, and histological examination significantly increase (x, culture; $P < 0.0001$; RUT, $P < 0.0001$; histology, $P < 0.0001$ [chi-square test]).

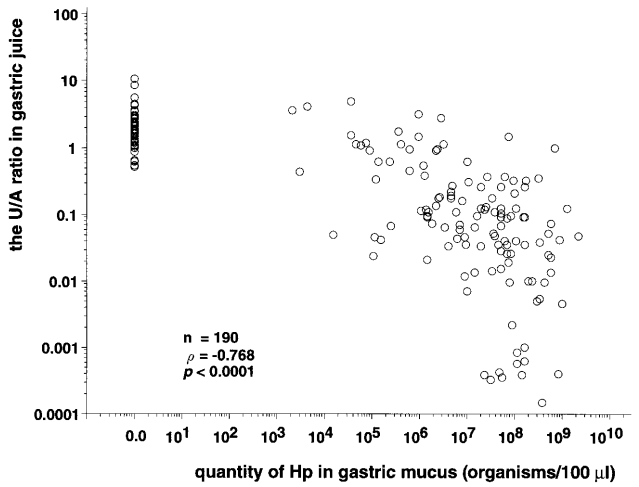


FIG. 6. Relationship between the quantity of *H. pylori* (Hp) in gastric mucus and the U/A ratio in gastric juice. As the quantity of *H. pylori* in gastric mucus increases, the U/A ratio in gastric juice significantly decreases ($\rho = -0.768$; $P < 0.0001$).

amount of *H. pylori* organisms to allow detection, since the distribution of *H. pylori* in the stomach is patchy (8, 19). Aspirated gastric juice contains gastric mucus in which *H. pylori* can be detected (16) and can usually be easily obtained in relatively large volumes. Thus, *H. pylori* detection from gastric mucus by PCR is, we believe, very sensitive and does not yield false-negative results because of sampling errors. The cPCR method that we used made it possible to determine the quantity of *H. pylori* in gastric mucus. As opposed to that in biopsy samples, the quantity of *H. pylori* in gastric mucus obtained from aspirated gastric juice reflects the global level of infection in the stomach. Quantitative determination of *H. pylori* in gastric mucus by cPCR amplification thus has the potential to be a rapid, highly sensitive, and specific tool for the laboratory diagnosis of *H. pylori* infection.

Some patients with negative findings for *H. pylori* in the RUT, bacterial culture, or histological examination were shown to be infected with *H. pylori* by the cPCR method. Notably, the smaller the quantity of *H. pylori* in the mucus, the lower the rates of positivity of RUT, histological examination, and bacterial culture. This finding suggests that RUT, histological examination, and bacterial culture may require relatively large quantities of *H. pylori* if positive findings are to be obtained. These results might be related to problems with the use of biopsy samples for RUT, histological examination, and bacterial culture, such as sampling errors, technical difficulties, and so on.

The UBT has been reported to reflect the quantity of *H. pylori* in the stomach, as assessed by histological examination (6). Our results support this finding. The correlation between the number of *H. pylori* organisms in the gastric mucus measured by our cPCR method and the results of ^{13}C-UBT suggests that both methods are useful for the quantitative evaluation of *H. pylori* infection in the stomach. However, one problem that we encountered with the use of the ^{13}C-UBT was in establishing an appropriate cutoff value. The ^{13}C-UBT may give a false-positive result if the patient's mouth is not completely rinsed after the administration of [^{13}C]urea, if the patient belches or sighs deeply, and so on. Although positive results were obtained by the ^{13}C-UBT, these were not always true-positive results, especially when the value was not particularly high. The two patients in the present study in whom only the ^{13}C-UBT gave positive results (Δ value > 6.0; culture, negative; histological examination, negative; RUT, negative; and cPCR result, 0 organisms per 100 μl of gastric mucus) were thought to be false positives.

There have been many reports concerning the detection of *H. pylori* by PCR methods. The levels of sensitivity of these techniques are very high, and they are thought to have a threshold of detection of no more than 100 *H. pylori* organisms (4, 18). However, this high degree of sensitivity increases the risk of false-positive findings because of contamination in clinical cases. Our findings suggest that when the stomach is infected with *H. pylori*, the quantity of *H. pylori* present in the stomach is relatively large and is never on the order of 100 organisms. Therefore, quantitative determination of *H. pylori* can distinguish true-positive findings from false-positive findings, since the quantity of *H. pylori* in the case of contamination that we experienced was very small.

A major problem with regard to conventional PCR detection of *H. pylori* is that when the result of PCR is negative, it is possible that PCR amplification was suppressed by a specific inhibitor which may be included in the sample DNA solution, preventing positive findings from being obtained. By our cPCR method, when amplification is suppressed by a specific inhibitor, not only *H. pylori* DNA bands (132 bp) but also sDNA bands (66 bp) are undetectable. Therefore, our cPCR method is thought to be able to distinguish true-negative findings from false-negative findings. In our experience, when neither sDNA bands nor *H. pylori* DNA bands are detected, decreasing the amount of *H. pylori* DNA solution added to the master mix in order to decrease the concentration of the specific inhibitor permits both *H. pylori* DNA bands and sDNA bands to be amplified. This finding also suggests that a specific inhibitor may sometimes be included in the sample DNA solution.

In the present study, we quantified *H. pylori* in gastric mucus using cPCR. This method can also be applied to the quantitative analysis of *H. pylori* in any biological sample, such as gastric mucosal tissue, saliva, and so on, as long as DNA can be extracted from the sample.

H. pylori is now recognized to be an important cause of gastroduodenal disease, and many kinds of anti-*H. pylori* treatments will be tested in the future. Precise evaluation of *H. pylori* infection is therefore of great importance. Our new method appears to be a very reliable test for *H. pylori* infection.

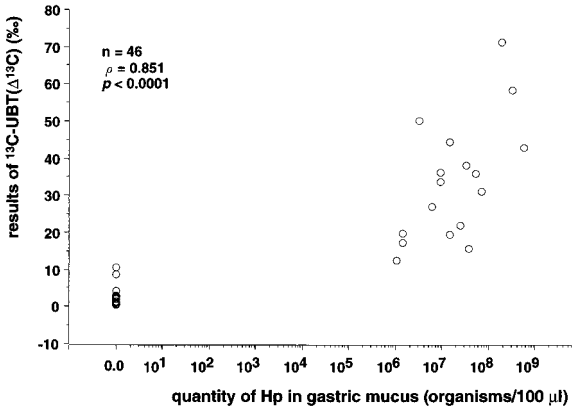


FIG. 7. Relationship between the quantity of *H. pylori* (Hp) in gastric mucus and results of ^{13}C-UBT. As the quantity of *H. pylori* in gastric mucus increases, the delta per mil change increases significantly ($\rho = 0.851$; $P < 0.0001$).

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