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Novel selective medium for the isolation of *Helicobacter pylori* and its prevalence in oral cavities

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Abstract

*Helicobacter pylori* is associated with gastrointestinal tract diseases; however, it is unclear whether this organism inhabits the oral cavities of humans.

The purpose of the present study was to develop selective media (OHPSM) for the isolation of *H. pylori* and investigate the prevalence of this organism in human oral cavities. *H. pylori* reference strains grew well on OHPSM. Among all subjects (n = 200), the number of *H. pylori* positive saliva samples was only 3 (1.5%). *H. pylori* in positive saliva samples was detected at 0.000013% of total bacteria. The mean numbers of *H. pylori* in those samples were 1.06 × 10 CFU/ml. All of *H. pylori* isolates from the same subject showed the identical genotype on AP-PCR using OPA-07. These results indicate that *H. pylori* is not a part of normal oral flora and is transient resident.

Key words: *Helicobacter pylori*, Selective medium, Oral cavity

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1. Introduction

At present, the genus *Helicobacter* consists of 54 species (https://lpsn.dsmz.de/search?word= Helicobacter). Among the genus *Helicobacter*, *Helicobacter pylori* is a Gram-negative rod which colonizes gastric mucosa in humans1). *H. pylori* infection is associated with gastrointestinal tract diseases including chronic gastritis, peptic ulcer, mucosa-associated lymphoproliferative disorders, and gastric cancer2–4). The global prevalence of *H. pylori* infection is more than 50%. This prevalence may vary significantly within and among countries, according to geography, ethnicity, age and socioeconomic factors5–6). It has been reported that *H. pylori* infection rate is up to 70% in developing countries, while the rate in developed countries and regions, such as Australia and Western Europe, are only 25% and 28%, respectively7). However, *H. pylori* infection rate in Japanese people over 40 years older is higher than 70%, and is clearly higher than other developed countries8). Socioeconomic factors also explain a significant proportion of the difference in *H. pylori* prevalence. In the third National Health and Nutrition Examination Survey conducted in the United States, a 25% prevalence of *H. pylori* infection was found in
children and young adults between 6 and 19 years. In the American population, prevalence was 42%; prevalence was higher in children of a low socioeconomic status, in those whose mothers had a lower education level, and in those living in crowded conditions. O’Rourke et al. evaluated the H. pylori infection in Mexican and American children living on both sides of the Rio Grande (a river that is dividing both countries): a slightly higher prevalence was observed in Mexicans compared to Americans. In a similar study by Goodman et al., they found a prevalence of 74% and 56% of Mexican and American women, respectively. The relationship between socioeconomic status and H. pylori infection has been reported too in countries such as Bolivia. Epidemiologic studies have shown evidence that most infections are acquired in childhood, but the specific age of acquisition and the factors associated with its persistence are not clear. In developing countries, 70 to 90% of the population become infected during childhood; in developed countries, a smaller percentage (>10%) of children become infected, and the prevalence of infection increases with age.

H. pylori infection mainly spreads through consumption of contaminated food and drinks or is transmitted orally. Several studies have evaluated the oral cavity, and H. pylori has been detected in dental plaque and saliva. It is known that the presence of H. pylori in the oral cavity is one of the main causes of the reappearance of gastric H. pylori infection and that treatment of oral infection significantly increases eradication of H. pylori infection in the stomach. Nevertheless, the role of dental plaque as an extra gastric reservoir in H. pylori transmission is controversial. Also, the oral H. pylori infection has been related to oral cavity problems, such as gingivitis, periodontitis, and recurrent aphthous stomatitis (RAS). Nisha et al. showed a highly significant association was found between periodontal disease and colonization of H. pylori in dental plaque.

Regrettably, it currently remains unclear whether H. pylori is part of the normal oral flora. In our previous pilot study, we tried to detect this organism from the oral samples using commercial selective media for H. pylori. However, it was impossible to identify this organism, because those media were not able to completely inhibit the growth of oral bacteria whose number was reported to exceed 600 species. Thus, a suitable selective medium is needed to assess the prevalence of oral H. pylori in gingivitis, periodontitis, and recurrent aphthous stomatitis in the oral cavities. The monitoring of H. pylori levels in the oral cavities might be useful for the diagnosis and prevention of H. pylori infectious disease.

The purpose of the present study was to develop selective media for the isolation of H. pylori from various oral samples, assess the prevalence of this organism in the oral cavity, and investigate whether H. pylori is a part of normal oral flora or is transient resident.

2. Materials and methods

2.1. Bacterial strains, culture conditions, and base medium for selective medium

All bacterial strains used in the present study are listed in Table 1 and 2. H. pylori and strains other than anaerobic bacteria were maintained by cultivating them on Columbia blood agar base (Oxoid Ltd., Hants, UK) supplemented with 7% horse blood (Columbia 7% blood agar). These organisms were cultured at 37°C for 48 h in an atmosphere of 5% CO2 in a CO2 incubator (NAPCO Model 5400; Precision Scientific, Chicago, IL, USA). The anaerobic bacteria (i.e., Veillonella parvula and Fusobacterium nucleatum) used in the present study were maintained by cultivating them on anaerobic blood agar (CDC), which has a Tryptic soy agar (Becton, Dickinson and Co., Sparks, MD, USA) base supplemented with vitamin K1 (10 µg/ml), hemin (5 µg/ml), L-cysteine (800 µg/ml), 0.5% yeast extract, and 5% sheep blood. These organisms were cultured at 37°C for 48 h in an anaerobic jar with a gas pack system (AnaeroPack®, Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan).

In our pilot study, the most suitable base me-
medium for the growth of *H. pylori* on selective medium was investigated. 18 mL of Columbia 7% blood agar exhibited the best growth of *H. pylori*, compared with other test media (data not shown). Therefore, 18 mL of Columbia 7% blood agar was determined for the base medium for the selective medium.

### 2.2. Susceptibility tests

Preliminary studies of antibiotic selection were also performed using disk susceptibility tests (Sensi-Disk, Becton Dickinson Co., MD, USA). The microbroth dilution method was used for susceptibility testing\(^{26}\).

2.3. Recovery of *H. pylori* and other representative oral bacteria

The recoveries of the *H. pylori* reference strains and other representative oral bacteria were calculated as CFU/ml on selective medium and compared with those on Columbia blood agar for total cultivable bacteria. All bacterial strains used in the present study are listed in Table 1 and 2.

*H. pylori* reference strains and other bacterial strains, except anaerobic bacteria, were pre-incubated in BHI broth supplemented with 10% serum at 37℃ overnight in an atmosphere of 5% CO\(_2\) in a CO\(_2\) incubator. Anaerobic bacteria were pre-incubated in Tryptic soy broth (Becton, Dickinson and Co., Sparks, MD, USA) sup-

| Table 1 Recovery of *H. pylori* and other bacteria on Columbia blood and OHPSM |
|---------------------------------|----------|----------|----------|-----------|
| Strain                          | Columbia blood | HPSM       | Recovery, % |
|                                 | CFU/ml, ×10\(^8\) | CFU/ml, ×10\(^8\) |         |
| *Helicobacter pylori*           |            |            |          |
| JCM 12093                       | 3.9 ± 0.2\(^a\) | 3.8 ± 0.3 | 97.7      |
| JCM 12095                       | 6.9 ± 0.2 | 6.8 ± 0.3 | 98.1      |
| JCM 12096                       | 4.5 ± 0.3 | 4.4 ± 0.2 | 97.3      |
| JCM 12097                       | 3.2 ± 0.1 | 3.2 ± 0.2 | 99.3      |
\(^a\)Ave ± SD.

| Table 2 Growth of other representative oral bacteria on Columbia blood and OHPSM |
|---------------------------------|----------|----------|-----------|
| Strain                          | Columbia blood | OHPSM       | Recovery, % |
|                                 | CFU/ml, ×10\(^8\) | CFU/ml, ×10\(^8\) |         |
| *Streptococcus oralis*          | ATCC 35037 | 4.9    | 0         |
| *Streptococcus salivarius*      | ATCC 10557 | 1.6    | 0         |
| *Streptococcus anginosus*       | ATCC 10557 | 3.4    | 0         |
| *Streptococcus mutans*          | ATCC 33397 | 4.8    | 0         |
| *Actinomyces naeslundii*         | NCTC 10449 | 0.3    | 0         |
| *Actinomyces oris*              | ATCC 12014 | 0.2    | 0         |
| *Actinomyces israelii*          | ATCC 27044 | 0.5    | 0         |
| *Corynebacterium matruchotii*   | ATCC 14266 | 0.4    | 0         |
| *Corynebacterium durum*         | ATCC 33449 | 0.7    | 0         |
| *Rothia dentocariosa*           | JCM 3067  | 0.6    | 0         |
| *Rothia mucilaginosa*           | JCM 10910 | 0.7    | 0         |
| *Rothia aeria*                  | JCM 11412 | 1.3    | 0         |
| *Veillonella parvula*           | ATCC10790 | 2.8    | 0         |
| *Fusobacterium nucleatum*       | ATCC22586 | 4.6    | 0         |
| *Neisseria sicca*               | ATCC 29256 | 0.4    | 0         |
plemented with vitamin K₁ (10 µg/ml), hemin (5 µg/ml), and 0.5% yeast extract at 37°C overnight under anaerobic conditions. Ten-fold dilutions of cultures were made in 0.9 ml of Tris-HCl buffer (0.05 M, pH 7.2) and aliquots of 0.1 ml were spread onto the test media. The plates on which bacteria, except anaerobic bacteria, were inoculated were cultured at 37°C for 5 days in an atmosphere of 5% CO₂ in a CO₂ incubator, and those on which anaerobic bacteria were inoculated were cultured at 37°C for 5 days under anaerobic conditions. After cultivation, the number of CFU/ml was counted.

2.4. Clinical samples

Two hundred volunteers (44 men, 56 women; mean age 45 years, range 8–78 years) participated in the present study. They had no systemic disease and received no antibiotic therapy for at least 3 months.

Paraffin-stimulated whole saliva samples were collected in a sterile microcentrifuge tube. All samples were dispersed by sonication for 30 s in an ice bath (50 W, 20 kHz, Astrason® System model XL 2020, NY., USA). Portions (100 µl) of appropriate dilutions of these samples were inoculated on three kinds of selective medium plates, i.e. selective medium developed in this study, and two commercial selective media for H. pylori, which were Pylori agar plates (bio-Méieux, Marcy-l’Étoile, France) and BD™ Helicobacter Agar. Modified (Becton Dickinson co., ltd., Heidelberg, Germany).

Selective medium plates were cultured at 37°C for 5 days in an atmosphere of 5% CO₂ in a CO₂ incubator. After cultivation, the number of CFU/ml on each selective medium was calculated and compared. This study was approved by the Ethics Committee of Nihon University School of Dentistry at Matsudo, Japan (EC 17-011).

2.5. Identification of H. pylori species isolated from clinical samples

ALL colonies that grew on the selective medium plate per subject were isolated and subcultured, and their identity was then confirmed by a polymerase chain reaction (PCR) analysis.

2.6. Design of species-specific primers for H. pylori

The design of species-specific primers for H. pylori was performed as follows. Helicobacter acinonychis, Helicobacter ceto rum, Helicobacter suis, Helicobacter bizzozeronii, and Helicobacter felis exhibit the closest phylogenetic association with H. pylori. The 16S rRNA sequences of H. pylori (accession no. U01330), H. acinonychis (accession no. AY596214), H. cетorum (accession no. AF292378), H. suis (accession no. EF204589), H. bizzozeronii (accession no. Y09404), and H. felis (accession no. M57398) were obtained from the DNA Data Bank of Japan (DDBJ; Mishima, Japan), and multiple sequence alignment analyses were performed using the CLUSTAL W program; i.e., the 16S rRNA sequences of four species were aligned and analyzed. Homologies among the primers selected for H. pylori were confirmed by a BLAST search.

2.7. Development of a PCR method for identifying H. pylori using designed primers

A PCR method for identifying H. pylori using the designed primers was developed as follows. Bacterial cells were cultured in a BHI broth supplemented with 10% horse serum 37°C overnight in an atmosphere of 5% CO₂ in a CO₂ incubator, and 1 ml of the sample was then collected in a microcentrifuge tube and resuspended at a density of 1.0 McFarland standard (approximately 10⁷ CFU in 1 ml of sterile distilled water). A total of 3.6 µl of the suspension was then used as a PCR template. The detection limit for PCR was assessed by serially diluting known numbers of bacterial cells in sterile distilled water and then subjecting each suspension to PCR. The multiplex PCR mixture contained 2 µM of each primer, 10 µl of 2× MightyAmp Buffer Ver. 3 (Takara Bio Inc., Shiga, Japan), 0.4 µl of MightyAmp DNA Polymerase (Takara), and 5.6 µl of the template in a final volume of 20 µl. PCR was performed in a DNA thermal cycler (Applied Biosystems 2720 Thermal Cycler; Applied Biosystems, Carlsbad, CA). PCR conditions included an initial denaturation step at
98°C for 2 min, followed by 30 cycles consisting of 98°C for 10 s and 68°C for 1 min. PCR products were analyzed by 2.0% agarose gel electrophoresis before being visualized by electrophoresis in 1 × Tris-borate-EDTA on a 2% agarose gel stained with ethidium bromide. A 100-bp DNA ladder (Takara Biomed, Shiga, Japan) was used as a molecular size marker.

### 2.8. Genotyping using AP-PCR analyses

Arbitrarily primed PCR (AP-PCR) analyses for genotyping of *H. pylori* isolates were performed as follows. Subcultured isolates were suspended in 1.0 McFarland standard in 100 μl of distilled water, and 5.6 μl of the suspension was used as a template for AP-PCR. AP-PCR conditions and OPA-07 primer used in this study were performed as described previously. OPA-07 (5'-GAAACGGGTG-3') produced clear and reproducible DNA bands in the preliminary study (data not shown) and was thus used in subsequent studies. Briefly, PCR mixture contained 0.2 μM of OPA-07 primer, 10 μl of 2 × MightyAmp Buffer Ver. 3 (Takara Bio Inc., Shiga, Japan), 0.4 μl of MightyAmp DNA Polymerase (Takara) and 5.6 μl of the template in a final volume of 20 μl. PCR was carried out in a DNA thermal cycler (Applied Biosystems 2720 Thermal Cycler). PCR running conditions included an initial denaturation step at 98°C for 2 min, followed by 35 cycles consisting of 94°C for 1 min, 32°C for 1 min and 72°C for 2 min, and a final extension at 72°C for 5 min. PCR products were analyzed by 1.3% agarose gel electrophoresis and visualized by gel staining with ethidium bromide. A 100-bp DNA ladder was used as a molecular size marker (Takara Biomed).

### 3. Results

#### 3.1. Development of selective medium

**3.1.1. Susceptibility to antibiotics**

*H. pylori* was more resistant to colistin than oral Gram-negative cocci, such as *Neisseria* and *Veillonella* species. The minimal inhibitory concentration (MIC) of colistin for *H. pylori* was 50 μg/ml. Oral Gram-negative cocci were sensitive to 7.5 μg/ml of colistin. *H. pylori* was more resistant to bacitracin and vancomycin than oral *streptococcus* species, oral *Corynebacterium* species, and oral *Actinomyces* species. The MIC of bacitracin and vancomycin for *H. pylori* was 1,000 μg/ml. Oral *streptococcus* species, oral *Corynebacterium* species, and oral *Actinomyces* species were sensitive to 75 μg/ml of bacitracin and vancomycin. *H. pylori* was more resistant to Trimethoprim-sulfamethoxazole (TS) combination than oral Gram-negative rods, such as *Leptotrichia, Aggregatibacter, Fusobacterium, Porphyromonas*, and *Prevotella* species. The MIC of TS combination for *H. pylori* was 200 μg/ml. Oral Gram-negative rods were sensitive to 15 μg/ml of TS combination.

#### 3.1.2. Composition of the new selective medium

The novel selective medium, designated oral *H. pylori* selective medium (OHPSM), was composed of the following (per liter): 39 g of Columbia blood agar base, 70 ml of horse blood, 75 mg of bacitracin, 75 mg of vancomycin, 7.5 mg of colistin, 15 mg of TS combination, and 5 mg of amphotericin B. Horse blood and antibiotics, i.e., bacitracin, vancomycin, colistin, TS combination, and amphotericin B were added after the base medium had been sterilized and cooled to 50°C.

#### 3.2. PCR method for identifying *H. pylori*

**3.2.1. Primer design**

The specific primer set covering the upstream region of the 16S rDNA sequence of *H. pylori* was designed in the present study (Table 3). The amplicon size of *H. pylori* was 869 bp.

**3.2.2. Detection limit**

A PCR method was used to identify the *H. pylori*-amplified DNA fragment of the expected size for this organism. The detection limit was assessed in the presence of titrated bacterial cells, and the detection sensitivity of the PCR assay was 5 CFU per PCR template (5.6 μl) for the *H. pylori*-specific primer set with the JCM 12093 strain (Fig. 1).
3.2.3. Assay of *H. pylori* and representative oral bacteria

The PCR method used to identify *H. pylori* produced positive bands from the *H. pylori* reference strain JCM 12093 (Fig. 1). Some *Streptococcus*, *Actinomyces*, *Neisseria*, *Corynebacterium*, *Rothia*, *Veillonella*, *Fusobacterium*, *Aggregatibacter*, and *Staphylococcus* species were used as representative oral bacteria in PCR using the designed primer set. No amplicons were produced from any of the representative oral bacteria (data not shown).

### 3.3. Recovery of *H. pylori* and inhibition of other representative oral bacteria on selective medium

Table 1 shows the recovery of the *H. pylori* reference strain JCM 12093, JCM 12095, JCM 12096, and JCM 12097 on OHPSM relative to Columbia 7% blood agar. The growth recoveries of the *H. pylori* reference strains were between 97.3 and 99.3% (average 98.1%) that on Columbia 7% blood agar.

Table 2 also shows the inhibition of other representative oral bacteria on OHPSM relative to Columbia 7% blood agar. The growth of other representative oral bacteria was markedly inhibited on the selective medium, OHPSM.

#### 3.4. Clinical examination

Detection frequencies of *H. pylori* in saliva samples obtained from two hundred subjects with 3 selective media, are shown in Table 4. With OHPSM which was developed in this study, *H. pylori* was detected in only 3 samples (1.5%). The mean number of *H. pylori* in *H. pylori* positive subjects were $1.06 \times 10^3$ CFU/ml. The mean numbers of other bacteria on OHPSM were $1.5 \times 10^2$ CFU/ml. However, with two commercial selective media (Pylori agar plates and BD™ Helicobacter Agar, Modified), *H. pylori* was not able to be detected at all. As a reason for that, two commercial selective media was not able to inhibit the growths of oral bacteria other than *H. pylori*. Fig. 2 shows the comparison of 3 selective media which were inoculated with a *H. pylori* positive saliva sample. OHPSM was able to distinguish *H. pylori* from other bacteria easily, but Pylori agar plates and BD™ Helicobacter Agar, Modified, were not, because of the superior growth of other bacteria.

In the first isolation, *H. pylori* colonies on OHPSM commonly had a smooth and circular appearance, and those margins were fuzziness. The colony color and average colony size of *H. pylori* on OHPSM were white and 1.3 mm in diameter, respectively (Fig. 3A). Fig 3B shows Gram stain image of *H. pylori* isolate. Cells exhibited Gram-negative, and had a helical, curved, or straight unbranched appearance and rounded ends.
3.5. **Comparison of genotypes among H. pylori isolates from the positive samples**

Fig. 4 shows the result of genotyping of three *H. pylori* isolates each from three positive subjects, by AP-PCR using OPA-07 primer. The AP-PCR patterns of *H. pylori* isolates were absolutely different among the subjects, but identical in the same subject.
frequently applied for diagnosis, culture method is needed to detect an early infection when an antibody response might be still absent. Furthermore, culture method is needed to determine the antimicrobial susceptibility pattern of individual strains. Several selective media have been used for the isolation of *H. pylori* which is not extremely fastidious, but very sensitive to oxygen, since it is a microaerophile, and requires an incubation period of 3 to 5 days \(^{28}\). Microbiologic culture of *H. pylori* is the “gold standard” for diagnosis in a patient with suspected infection. Culture method undoubtedly constitutes the most specific way to establish the diagnosis of *H. pylori* infection, though its sensitivity has been reported to vary greatly among laboratories. The provision of a good selective medium for *H. pylori* will help establish the role of this organism in the aetiology of not only gastrointestinal tract diseases but also oral cavity problems, such as gingivitis, periodontitis \(^{21, 22}\), and RAS \(^{23}\).

Of several selective media for *H. pylori*, Pylori agar plates and BD™ Helicobacter Agar, Modified, are frequently used in the clinical laboratories all over the world. In our previous pilot study, we tried to detect this organism from the oral samples using two commercial selective media for *H. pylori* described above. However, it was impossible to identify this organism, because those media were not able to completely inhibit the growth of oral bacteria whose number was reported to exceed 600 species \(^{25}\).

It currently remains unclear whether *H. pylori* is part of the normal oral flora. A suitable selective medium and reliable identification method are needed in order to assess the prevalence of oral *H. pylori* involved in oral infectious diseases. In the present study, *H. pylori* reference strains were more resistant to bacitracin, vancomycin, colistin, and TS combination than other representative oral bacteria. The growth of oral bacteria detected in the oral cavity was inhibited by the addition of 75 mg/L bacitracin, 75 mg/L vancomycin, 7.5 mg/L colistin, and 15 mg/L TS combination to Columbia 7% blood agar. *H. pylori* reference strains used in this study were JCM 12093 strain, JCM 12095 strain, JCM 12096 strain, and JCM 12097 strain. JCM 12093 strain was type strain of *H. pylori*, and was isolated from Australian patient with gastritis. JCM 12095 strain, JCM 12096 strain, and JCM 12097 strain were isolated from gastric mucosa of a French patient with gastritis, a healthy pig-tailed macaque, and a British patient with gastritis, respectively. All of the *H. pylori* reference strains grew well on the new selective medium, designated as OHPSM, while the growth of other bacteria was markedly inhibited (Table 1 and 2). Moreover, OHPSM was able to easily distinguish *H. pylori* by its characteristic colony morphology. Because OHPSM exhibited high growth recoveries in all of the *H. pylori* reference strains derived from various specimens and high selectivity, it was indicated that OHPSM was useful for detecting this organism from various oral samples, without showing false-positive or false-negative.

In the present study, *H. pylori* was detected in only 3 samples (1.5%) by a culture method using the selective medium, i.e. OHPSM. However, with two commercial selective media, *H. pylori* was not able to be detected at all. Moreover, OHPSM was able to distinguish *H. pylori* from other bacteria easily, but two commercial selective media were not, because of the superiority of growth of other bacteria. There are reports showing high detection rate from oral cavity \(^{29}\).
but the isolates were not identified by DNA based method such as PCR. Some studies have reported very low detection rate from oral cavity\textsuperscript{30-32}. Also, the attempts to detect \textit{H. pylori} using culture method from dental plaque or saliva from Swedish patients who had culture positive gastric biopsy specimen, were not successful\textsuperscript{33}. As well as previous some studies, the present results indicated that \textit{H. pylori} was not a part of normal oral flora. Accordingly, the monitoring of \textit{H. pylori} presences and its levels may not be useful as a clinical indicator for the diagnosis of gastrointestinal tract diseases.

PCR is used not only for the detection of bacterium but also for characterization of pathogenic genes and specific mutations associated with antimicrobial resistance. The conserved genes used for detection of \textit{H. pylori} are urease operon: ureA and glmM, also known as ureC, or the 16S rRNA, 23S rRNA and HSP60 genes\textsuperscript{34}. It is necessary to known the DNA sequence of the target gene in as many strains of \textit{H. pylori} and other related bacterial species as possible for designing specific primers. PCR based studies on the detection of \textit{H. pylori} from oral cavity have shown variable results since many uncharacterized bacteria closely related to \textit{H. pylori} are present in oral cavity and the pit of gastrointestinal tract, the results should be considered positive when at least two targeted conserved genes specific to \textit{H. pylori} give expected amplification. The sensitivity of PCR is able to be increased by performing nested or semi-nested approaches. However, such approaches might increase the possibility of false positive results caused by crossover contamination as well as the detection of DNA from dead bacteria\textsuperscript{30}. It might be difficult to directly detect \textit{H. pylori} from the specimens in which a lot of bacteria are contained. Accordingly, the present study tried to detect \textit{H. pylori} with culture method using the selective medium, and then the isolates were identified with a PCR method. In the present study, we designed species-specific primers to identify \textit{H. pylori} with a PCR method. These primers were able to distinguish \textit{H. pylori}, and did not react with representative oral bacteria. Moreover, the PCR method in the present study directly uses bacterial cells with MightyAmp DNA Polymerase Ver. 3 (Takara) and is completed within approximately 2 hours.

In this study, all of \textit{H. pylori} isolates from the same subject showed the identical genotype on AP-PCR using OPA-07. Also, \textit{H. pylori} in positive saliva samples was detected at 0.000013% of total bacteria. The mean numbers of \textit{H. pylori} in those samples were $1.06 \times 10^5$ CFU/ml. These results indicated that oral cavity may be only transitory reservoir as \textit{H. pylori} is coming here due to regurgitation or vomiting.

We developed a selective medium, designated OHPSM, to isolate \textit{H. pylori} in the oral cavity of humans. Since OHPSM is highly selective for \textit{H. pylori}, it will be useful for assessing the distribution and role of this organism at various locations in humans. The selective medium (OHPSM) and our PCR method as isolation and identification methods, respectively, for \textit{H. pylori} may contribute to make clear the role of this organism in the aetiology of not only gastrointestinal tract diseases but also oral cavity problems, such as gingivitis, periodontitis, and RAS.

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References

7) Rothenbacher D, Brenner H: Burden of \textit{Helicobacter pylori} and \textit{H. pylori}-related diseases in developed countries: recent developments and future implications, Mi-


