Detection of *Helicobacter pylori* DNA in inflamed dental pulp specimens from Japanese children and adolescents

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INTRODUCTION

*Helicobacter pylori* is a Gram-negative microaerophilic bacterium responsible for chronic gastritis, peptic ulcers and gastric cancer (Fennerty, 1994). Details regarding transmission and infection source are controversial, and it has been suggested that most infections are acquired in childhood, mainly via the oral cavity (Prasanthi et al., 2011). *H. pylori* has been isolated from, and its DNA detected in, gastric biopsy and faecal samples (Momtaz et al., 2011). Various PCR methods have been used to detect *H. pylori* DNA in oral specimens with various detection rates reported. Such disparity in detection rates complicates the estimation of the true infection rate of *H. pylori* in the oral cavity. In the present study, we constructed a novel PCR system for *H. pylori* detection and used it to analyse oral specimens. Firstly, the nucleotide alignments of genes commonly used for *H. pylori* detection were compared using the complete genome information for 48 strains registered in the GenBank database. Candidate primer sets with an estimated amplification size of approximately 300–400 bp were selected, and the specificity and sensitivity of the detection system using each primer set were evaluated. Five sets of primers targeting *ureA* were considered appropriate, of which a single primer set was chosen for inclusion in the PCR system. The sensitivity of the system was considered appropriate and its detection limit established as one to ten cells per reaction. The novel PCR system was used to examine *H. pylori* distribution in oral specimens (40 inflamed pulp tissues, 40 saliva samples) collected from Japanese children, adolescents and young adults. PCR analysis revealed that the detection rate of *H. pylori* in inflamed pulp was 15 %, whereas no positive reaction was found in any of the saliva specimens. Taken together, our novel PCR system was found to be reliable for detecting *H. pylori*. The results obtained showed that *H. pylori* was detected in inflamed pulp but not saliva specimens, indicating that an infected root canal may be a reservoir for *H. pylori*.

Abbreviation: ATCC, American Type Culture Collection.

One supplementary figure and one supplementary table are available with the online Supplementary Material.
PCR amplification was performed in a total volume of 20 μl. Genomic DNA extraction. H. pylori genomic DNA was extracted using a method reported for Gram-negative periodontitis-related bacteria (Amano et al., 1999). Briefly, 5 ml cultures of \( H. pylori \) were collected and washed by centrifugation, and the cells were resuspended in 250 μl 10 mM Tris/HCl (pH 8.0) containing 100 mM NaCl and 1 mM EDTA. The cells were collected by centrifugation and lysed in 600 μl cell lysis solution (Qiangen) for 5 min at 80 °C. The cell lysate was incubated with 3 μl RNase (10 mg ml\(^{-1}\); Qiagen) for 30 min at 37 °C, after which 200 μl protein precipitation solution (Qiagen) was added and the sample vortexed vigorously for 20 s. Genomic DNA was extracted with 600 μl 2-propanol (Wako Pure Chemical Industries) and collected by centrifugation. The DNA pellet was washed with 70% ethanol (Wako), air-dried and dissolved in 100 μl Tris-EDTA buffer [10 mM Tris/HCl, 1 mM EDTA (pH 8.0)].

Construction of a PCR system for identification of \( H. pylori \). The complete genome sequences for 48 \( H. pylori \) strains were obtained from the GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/). Multiple alignments of the complete 16S rRNA, vacA, cagA, glnM (ureC) and ureA genes were performed using CLUSTAL W from the DNA Data Bank of Japan (http://clustalw.ddbj.hig.ac.jp/). The nucleotide alignments of at least 20 consecutive sequences consistent among all 48 strains were searched and selected as possible candidate primer sets for the detection of \( H. pylori \).

PCR amplification was performed in a total volume of 20 μl with 2 μl template DNA (20 μg ml\(^{-1}\)) and TaKaRa Ex Taq (Takara Bio) according to the supplier’s instructions. PCR amplification was performed in a thermal cycler (iCycler; Bio-Rad) with the following cycling parameters: initial denaturation at 95 °C for 4 min, followed by 30 cycles at 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, with a final extension at 72 °C for 7 min. The PCR products were fractionated in a 1.5% (w/v) agarose gel using Tris-acetate-EDTA buffer, stained with ethidium bromide (0.5 μg ml\(^{-1}\)) and visualized under UV illumination. A 100 bp DNA ladder (New England Biolabs) was used as the molecular size standard.

The specificity of primer sets for \( H. pylori \) was assessed using genomic DNA extracted from \( H. pylori \), \( H. pullorum \) and \( H. felis \). The sensitivity of the PCR assays was determined using titrated cultures of strain J99. The detection limits of the PCR assays for the five primers sets were determined using ten-fold serial dilutions of genomic DNA extracted from known numbers of bacterial cells.

### RESULTS

### Construction of a novel PCR system for identification of \( H. pylori \)

Multiple alignment analysis of the complete genome sequences from 48 \( H. pylori \) strains revealed that neither

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**Fig. 1.** Macroscopic images of representative oral samples. (a) Inflamed pulp specimens collected using sterile root canal instruments. (b) Saliva specimens collected in sterile plastic tubes.
vacA nor cagA genes contained sequences of $\geq 20$ consecutive nucleotides, while only one consecutive sequence ($\geq 20$ nt) was identified on the 16S rRNA and glmM genes. However, six sequences $\geq 20$ consecutive nt in length were identified in the ureA gene across all 48 strains (see Fig. S1, available in the online Supplementary Material). Consequently, these sequences were used in the design of five sets of primers, producing PCR products 300–400 bp in length (Table 1, Fig. 2a). All five primer sets produced amplicons consistent in size with the estimated 300–400 bp fragment for genomic DNA across all *H. pylori* strains, but were notably absent for *H. pullorum* and *H. felis* strains. These results demonstrated the high specificity of all primer sets for detecting *H. pylori*. In addition, all primer sets showed an appropriate level of sensitivity at approximately 1–10 c.f.u. per reaction (Fig. 2b). The primer set ureA-aF/ureA-aR was the most sensitive of all primer combinations and was therefore used for all further analyses.

**Detection of *H. pylori* in oral specimens**

Fig. 3 shows results from the analyses of clinical specimens using the primer set ureA-aF/ureA-aR. *H. pylori* was detected in 6 of 40 (15.0 %) root canal specimens (Table 2). Densitometry analyses estimated that positive clinical specimens contained approximately $10^2$–$10^3$ c.f.u. *H. pylori*. In contrast, none of the saliva specimens were positive for *H. pylori* using our PCR system (Table S1). All amplified PCR products were sequenced and the results were consistent with the targeted ureA sequences of *H. pylori*.

**DISCUSSION**

The development of molecular biological techniques has enabled the detection of *H. pylori* DNA in various specimens (Dunn et al., 1997), with a number of target genes, including 16S rRNA, vacA, cagA, glmM and ureA, proposed for use in PCR detection systems (Mapstone et al., 1993; Miyabayashi et al., 2000; Wang et al., 2002; Park et al., 2003; Smith et al., 2004). The complete genome sequences for a variety of *H. pylori* strains isolated worldwide have been reported (Furuta et al., 2011; Kawai et al., 2011). To our knowledge, this is the first study to successfully design primer sets based on the complete genome sequence of 48 diverse clinical strains.

Primers specific for the vacA and cagA genes are commonly used to detect *H. pylori* (Wang et al., 2002; Park et al.,

![Image](http://jmm.sgmjournals.org)

**Fig. 2.** Construction of a PCR system for the detection of *H. pylori*. (a) Schematic showing positions of primers designed in the present study. (b) Representative image showing assay sensitivity for the detection of *H. pylori* J99 using the ureA-aF and ureA-aR primer sets. Sensitivity was examined using titrated cultures containing $10^8$ cells ml$^{-1}$ from strain J99. M, 100 bp DNA ladder; N, sterile water.
Several studies have used molecular biological techniques to identify *H. pylori* DNA within the oral cavity (Prasanthi et al., 2011). However, it is unclear whether *H. pylori* can survive in the oral environment. *H. pylori* was previously classified as a *Campylobacter* species, which is known to be a major periodontopathic bacterium (Fennerty, 1994). Therefore, it is possible that the gingival sulcus may be a reservoir of *H. pylori* in the oral cavity.

*H. pylori* was detected at a higher frequency in periodontitis patients when compared with periodontally healthy subjects (Umeda et al., 2003). In fact, the depth of the gingival sulcus in healthy subjects was defined as 3 mm or less, whereas in periodontitis subjects, it was 4 mm or more (Umeda et al., 2003). However, it is widely known that periodontitis in children is quite rare, whereas gingivitis is commonly found in daily practice in primary dentition with a gingival sulcus depth of 1–2 mm (Jenkins & Papapanou, 2001). We speculate that *H. pylori* is present in various locations other than the periodontal pocket during childhood.

Dental caries is another major disease encountered in the field of dentistry, in which surface hard tissue is decalcified.
by the action of cariogenic bacteria (Lynch et al., 2013). When dental caries develops, destruction of the hard tissue extends into the pulp space, which is normally sterile and contains soft tissues such as nerves, blood vessels and connective tissues (Yip & Smales, 2012). Under these circumstances, it is possible that oral bacteria can enter the pulp space locale and aggravate inflammatory conditions; this is followed by pulp necrosis. Bacterial infection of the root canal generally consists of a mixture of microbial flora, including bacterial species such as \textit{Fusobacterium nucleatum}, \textit{Eubacterium alactolyticum}, \textit{Lactobacillus casei}, \textit{Peptostreptococcus micros} and \textit{Campylobacter rectus} (Matsuo et al., 2015).
et al., 2003). If infection spreads from affected teeth, it may cause purulent sinusitis, meningitis, brain abscess and infective endocarditis (Yu & Abbott, 2007). Recently, Helicobacter pylori was reported to be isolated from the root canals of endodontically-infected deciduous teeth (Hirsch et al., 2012). However, it is unclear whether H. pylori infection of pulp tissue is transient. In the present study, we investigated the distribution of H. pylori in saliva and inflamed pulp specimens. Although it is better to compare the distribution between dental pulp and saliva from the same individuals, there were a number of individuals who were not cooperative during dental treatment because they were too young. Some of them received root canal treatment with restraint and they could not provide saliva in sterile plastic tubes. Thus, we used 40 saliva specimens collected from another group of children. Although we analysed different individuals for dental pulp and saliva samples, our findings suggest that the detection rate of H. pylori in root canal specimens is higher than that in saliva specimens, indicating that the root canal may be a reservoir for H. pylori organisms.

ACKNOWLEDGEMENTS

The authors thank Professor Howard K. Kuramitsu, State University of New York at Buffalo, USA, for editing the manuscript. This study was supported by a Grant-in-Aid for Challenging Exploratory Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (grant no. 25670872).

REFERENCES


genotypes of *Helicobacter pylori* in stomach, saliva and dental plaque. 


