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Isolation and characterization of *Helicobacter pylori* recovered from gastric biopsies under anaerobic conditions

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ABSTRACT

Background and Aim: Helicobacter pylori can survive long incubation periods under anaerobic conditions, and should be possible to isolate under anaerobic conditions. Our aim was to isolate *H. pylori* in anaerobic conditions, from gastric biopsies of *H. pylori* infected patients.
Methods: We enrolled 27 patients with bleeding (erosive) gastritis (mean age 36.3 years, 55.6% male) from Hanoi, Vietnam. *H. pylori* status was confirmed by qPCR.
Results: H. pylori were recovered under anaerobic and micro-aerobic conditions from gastric biopsies in 16 patients. Anaerobic conditions yielded significantly higher *H. pylori* recovery rates than micro-aerobic conditions (81.3% vs. 31.3%, $P = 0.01$). *H. pylori* isolates were characterized by PCR for specific virulence markers and the genotypes were similar to those previously described in this region of the world.
Conclusions: H. pylori can be isolated under anaerobic conditions. These findings may provide new insight into the physiology of this human pathogen and help to identify the route of *H. pylori* transmission.

1. Introduction

Helicobacter pylori is the leading gastric pathogen that colonizes the human stomach and causes a spectrum of diseases such as chronic active gastritis, gastric ulceration, MALT lymphoma and gastric cancer among others (Kidd and Modlin, 1998; Warren, 1983; Marshall and Warren, 1984; Nomura et al., 1991; Biological Agents, 2012). The role of the bacteria as a human pathogen has been confirmed in many parts of the world (Go, 2002; Brown, 2000).

Helicobacter pylori is recognized as an inhabitant of the human gastric mucosa. The organism has been regarded as microaerophilic, as its optimal growth occurs in the presence of 5–15% oxygen (Goodwin and Armstrong, 1990). In general, primary cultures of *H. pylori* have less oxygen tolerance with a growth maximum at 3–7% O_2 (Marshall and Warren, 1984; Langenberg et al., 1984). Most studies with standardized atmospheres for culturing *H. pylori* have used 2–5% O_2, 5–10% (optimal closer to 10%) CO_2 and 0–10% H_2 (Andersen and Wadström, 2001; Kusters et al., 2006). Subcultures of *H. pylori* can rapidly be adapted to grow aerobically in a standard CO_2 mixture (18% O_2, 10% CO_2) in an incubator (Tompkins et al., 1994). In addition, *H. pylori* appear to survive in other gas environments such as 10% CO_2 alone or variations in oxygen levels (Goodwin and Armstrong, 1990; West et al., 1992).

There has been no consensus about the specific oxygen and carbon dioxide needs of *Helicobacter pylori.* Some investigators do not consider the bacteria a true micro-aerobic, as *H. pylori* is also a capnophile that grows equally well in vitro under micro-aerobic or aerobic conditions at high bacterial concentrations, and behaves like an oxygen-sensitive microaerophilic at low cell densities (Bury-Moné et al., 2006). In 1999, Yamaguchi reported that *H. pylori* strain IK1029 growing under microaerophilic condition was able to survive long incubation periods under anaerobic conditions (Yamaguchi et al., 1999). Furthermore, *H. pylori* has also been cultured from the feces (Thomas et al., 1992; Kelly et al., 1994) that form in the anaerobic large intestine. These reports suggest that *H. pylori* may grow under anaerobic conditions.

To determine whether *H. pylori* can grow in both an anaerobic and micro-aerobic environment, we incubated biopsy homogenates from *H. pylori* infected and not infected patients under each condition. We report here that *H. pylori* can be isolated as a primary culture under anaerobic conditions as well as under micro-aerobic conditions. We also report that the genotypes of the isolated *H. pylori*, characterized by traditional PCR, are consistent with the genotypes previously described in this region of the world, independent of culture conditions.

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2. Methods

2.1. Biopsy specimens

Patients enrolled in Buu Dien Hospital were interviewed and signed informed consent forms before undergoing endoscopic examination to monitor stomach lesions. Patients' consent forms were approved by a local committee at the Buu Dien Hospital. Gastric biopsies from 27 patients were taken from antrum (AB) and corpus (CB) and flash-frozen immediately in liquid nitrogen for further study without cryopreservative. In addition, gastric juice was collected from all patients and pH was measured using pH paper.

2.2. Microbiological Methods

2.2.1. Medium and Processing of Gastric Biopsy Samples

Non-selective medium commercial 5% Sheep Blood Agar was used for culturing *H. pylori*. Gastric biopsies were processed as previously described (Peek et al., 1995). The delay between the removal of the specimens from the freezer and the inoculation onto culture media did not exceed 2–4 h. Gastric biopsies were homogenized in 300 μL of sterile 1× PBS and then 30 μL of the suspension was spread on the surface of non-selective blood media. The rest of the biopsy homogenates were used for total genomic DNA purification.

2.2.2. Culture Condition

Plates were incubated at 37 °C under two different atmospheric conditions: anaerobic and microaerophilic, using the BD Gas Pak™ EZ gas generating container systems (BD Company, Sparks MD) (anaerobic) or the BD Campy container system (microaerophilic), respectively. *H. pylori* growth was monitored from days 3–7. Bacterial morphology was examined by Gram staining at a magnification of ×1000. Bacterial isolates recovered from anaerobic and microaerophilic conditions were confirmed as *H. pylori* on the basis of positive urease reaction, typical colony morphology (small, round colonies) and traditional PCR analysis for several bacterial genes including *cagA*, *vacA*, *hspA*, *23SrRNA* and the intergenic region between *jhp0153* and *jhp0152*. Furthermore, all *H. pylori* isolated recovered from anaerobic and microaerophilic conditions were plated again under the same atmospheric conditions to confirm their ability to grow in anaerobic and microaerophilic conditions.

2.3. Molecular methods

2.3.1. DNA purification

Total genomic DNA was purified from the biopsy homogenates using the DNeasy Blood and Tissue Kit (QIAGEN, Valencia CA). Bacterial DNA was purified from strains isolated under anaerobic and microaerobic conditions using the MBI GeneJet Genomic DNA purification Kit (Fermentas, Vilnius, Lithuania). Concentration of extracted purified DNA was determined using the NanoDrop 1000 (Thermo Scientific, Hanover Park IL).

2.3.2. PCR amplification

PCR amplification occurred with 1–2 μl (100–200 ng) of purified DNA from biopsies or bacteria. Each PCR reaction contained 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each dNTP, 25 pmol each primer, and 2.5 U of Taq polymerase. Temperature and step durations are included in Table 1. The reactions were conducted for 30–35 cycles using the Hybaid Thermocycler MBS 0.5G (Thermo Electron Corporation, Marietta OH). The PCR products were confirmed for size and purity on 1.5%–2% agarose gel run with 1× TAE buffer. The primers used in the analysis were shown in the Table 1.

2.3.2.1. cagA. The 3′end of the *cagA* gene was amplified with two primers, one of them degenerate (Panayotopoulou et al., 2007). The carboxyl terminal portion of CagA protein has multiple phosphorylation sites, and there is a clear difference in the number and type of phosphorylation sites between *H. pylori* strains isolated from Eastern and Western countries (Hatekeyama and Higashi, 2005). PCR conditions: 5-min hot start, 25 to 30 cycles as follows: 94 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min. For those strains that were *cagA* negative, we confirmed the lack of the pathogenicity island by empty-site PCR.

2.3.3. qPCR Methodology

A set of primer, and a TaqMan MGB probe specific for *H. pylori* were developed based on the *H. pylori 16S rRNA* gene. Forward primer: 5′-AGAGACTAAGCYCTCCAACAAC; Reverse primer: 5′-AATACTCATTGCGAAGGCGA; and Probe: 5′-TACGGGAGGCAGCAGT. Standards for total bacteria and *H. pylori* were prepared from the 16S rRNA, which were amplified from *H. pylori* strain 26,695 using the bacterial 8F/1510R primer (Gao et al., 2010). PCR products were cloned into the pGEMT easy vector (Promega, Madison, WI), and confirmed by sequencing. The qPCR-mix, with a total volume of 20 μl per well, consisted of 5 μM of forward and

Table 1
Primers used for PCR and qPCR.

Gene target	Primers	Reference	Temperature and step
cagA	5′-GTTAARAATRGTGTRAAYGG-3′ 5′-TTTAGCTTCTGATACCGC-3′	19	94 °C 30s, 56 °C 30s, 72 °C 1 min.
Empty-site	5′-CTCTTTTTGTGCCTTTGATTGAA-3′ 5′-CCAAATACATTTTGGCTAAATAAAC-3′	21	94 °C 30s, 54 °C 45 s, 72 °C 1 min.
hspA	5′GCTATCTGAAAATTTGATTTCTTTTGC-3′ 5′-TGCGCTATAGTTGTGTCGC-3′	23	94 °C 30s, 52 °C 30s, 72 °C 2 min.
Intergenic region	5′-GTGGCGCGTTTCTTGCAATACC-3′ 5′-AACTCGCTCAAAAACTCGGC-3′	24	94 °C 30s, 57 °C 30s, 72 °C 1 min.
23S rRNA	5′-CTCCATAAGAGCCAAAGCCC-3′ 5′-CCACAGCGATGTGGTCTC-3′	25	94 °C 60s, 60 °C 45 s, 72 °C 1 min.
VacA status			
vacA s-region	5′-TGAGTTGTTTGATATTGAC-3′ 5′-CAATCTGTCCAATCAAGCGAG-3′	22	94 °C 30s, 50 °C 45 s, 72 °C 45 s.
vacA m-region	5′-CTGCTTGAATGCGCCAAAC-3′ 5′-ATGGAAATACAACAAACACAC-3′	This study	94 °C 30s, 52 °C 30s, 72 °C 2 min.
qPCR			
Hp 16S rRNA	5′-AGAGACTAAGCYCTCCAACAAC-3 5′-AATACTCATTGCGAAGGCGA-3′	This study	
	HP probe 5′-CATTACTGACGCTGATTGYGC	This study	
Eubacteria 16 s rRNA	5′-CAGCAGCCGCGGTRATA-3 5′-CGYCAATTGBGBAADATTCC-3′	26	
	Eubacteria probe 5′-TACGGGAGGCAGCAGT-3′	26	