



ORIGINAL ARTICLE

Diagnosis of Genus *Helicobacter* through a hemi-nested PCR assay of 16S rRNA



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16S rRNA

Abstract The present study aimed to establish a genus-specific PCR-based assay to detect *Helicobacter* spp. using 16S rRNA gene as the target template. We designed the hemi-nested primers based on sequences of 16S rRNA gene of 34 types of *Helicobacter* species. The inclusivity, sensitivity, and specificity of the PCR assay using these primers were examined in three different models, comprising feces simulated samples, BLAB/c mice infection model and clinic patients samples. The detection sensitivity of *Helicobacter pylori*, *Helicobacter hepaticus* and *Helicobacter bilis* strains from feces simulated samples was all 102 CFU/ml. We successfully detected *H. hepaticus* and *H. bilis* in the liver, cecum and feces of experimentally infected mice. *H. pylori* was successfully detected in the feces samples from 3 patients infected with *H. pylori* while not in the feces samples from 3 healthy human. However, the C97/C05–C97/C98 PCR assay detected *H. pylori* in the 2 positive samples. Due to the PCR assay's excellent inclusivity, high sensitivity and specificity it may be used to detect the presence of *Helicobacter* spp.

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1. Introduction

A large number of studies confirmed that *Helicobacter* spp. have their share in the pathogenesis of diseases of the digestive system, ever since *Helicobacter pylori* was first recognized as an important factor in the genesis of gastritis, peptic ulcer and gastric adenocarcinoma in human (Parsonnet et al., 1991; Dunn et al., 1997). The number of *Helicobacter* spp. identified is increasing as more research is done in this field. With 34 *Helicobacter* species now formally named according to LPSN (<http://www.bacterio.net/h/helicobacter.html>), it is clear that

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Helicobacter species can infect human and various animal hosts, as well as colonize different anatomical regions of the gastrointestinal system (Fox, 2002; Recordati et al., 2009).

Culture of *helicobacter* organisms can be used to identify infected hosts. However, the bacteria are fastidious and initial in vitro isolation therefore requires special microaerophilic conditions and 5–7 days (Fox et al., 1994, 1995). Detection of *helicobacters* by culture is further complicated by the presence of normal flora, since the preferred site for isolation of the bacteria is the gastrointestinal tract. In addition to culture, the immunological assays are also important techniques. However, the difficulties to culture many fastidious *Helicobacter* species to obtain enough cells for the extraction of cell surface proteins are still a challenge. Therefore there is a need to develop an easy to use and reliable assay to detect and discriminate *helicobacters* (Wadstrom et al., 2009).

The association between the *Helicobacter* spp. and gastrointestinal diseases can also be determined by proving the presence of the *Helicobacter* spp. DNA in samples that are collected during the examinations. The procedure became simpler and more precise, which allowed an evolution of PCR in the sense of being more feasible, but also a sophisticated and reliable method that has proven its usefulness in dealing with the association. (Engstrand et al., 1992; Lin et al., 1995; Fox et al., 1998; Myung et al., 2000; Monstein et al., 2002). However, Poynter et al. reported that three out of six different commercial laboratories performing *helicobacter* testing on the same spiked fecal samples failed to detect and identify *Helicobacter hepaticus* due to the differences in the design of their PCR assays (Poynter et al., 2009). Bulajic et al. concluded that further investigation of *Helicobacter* spp. role in the development of malignancies and other diseases requires targeting of new genome sequences or design of more specific primers (Bulajic et al., 2012). The PCR subtypes used included nested PCR and single-round PCR. Nested PCR has been used frequently in recent years due to its higher specificity. The most sensitive and widely used method for detecting *helicobacter* infections is PCR targeting at a genus-specific and conserved region of 16S rRNA (Battles et al., 1995; Bulajic et al., 2012). In this study, we have tried to establish and optimize a hemi-nested PCR assay that would simultaneously detect and differentiate 34 of the formally named *helicobacters* from feces samples. The primers were designed based on the sequences of 16S rRNA.

2. Materials and methods

2.1. Strains and culture

H. pylori (ATCC43504), *H. hepaticus* (ATCC51448), *Helicobacter bilis* (ATCC43879) were obtained from the department of Gastroenterology in NanFang Hospital. *H. pylori* were cultured on Brucella agar. *H. hepaticus*, *H. bilis* and *Campylobacter jejuni* were cultured on selective *C. jejuni* agar (containing 10 mg/L vancomycin, 5 mg/L trimethoprim, and 2500 U/L polymyxin B), supplemented with 5–10% sterile defibrinated sheep blood. Plates were incubated for 3–7 days under microaerobic conditions at 37 °C at high humidity in microaerobic chamber (10%CO₂, 5%O₂, and 85%N₂). *Escherichia coli*, and *Enterococcus faecalis* were cultured in Columbia agar at 37 °C.

2.2. Genomic DNA extraction

Bacteria were cultured on plates and the cells were harvested and washed twice with 1 ml of PBS. Bacteria genomic DNA extraction was performed using Qiagen RNA/DNA Mini Kit (Qiagen Inc.) according to the manufacturer's instructions.

2.3. DNA extraction from feces and tissue

DNA was extracted from 220 mg of fecal and tissue specimen using the QIAamp DNA Mini stool Kit (Qiagen Inc.) according to the manufacturer's instructions. DNA content and purity were determined spectrophotometrically by measuring the A260/A280 optical density ratio.

2.4. The primers were analyzed using DNAMAN 8.0 and OLIGOU 7.0

2.4.1. PCR amplification

The hemi-nested universal Primers were designed with Primer Premier 6.0 on the basis of nucleotide sequence of 16S rRNA of all 34 type strains. Primary reactions used 1.0 µl of purified bacteria DNA (1 ng), or 5.0 µl of serially diluted suspensions of bacteria spiked stool DNA (25–100 ng) as the template in a total volume of 50 µl, unless otherwise stated. In the first amplification step Genomic DNA was amplified in a total volume of 50 µl containing PCR buffer (1X), 200 µM dNTP, 1.5 mM Mg²⁺, 0.2 mM primers (outer-F: 5' CTGGCGGCGTGCCTAATAC 3' and outer-R: 5' CTCACGACACGAGCTGAC 3') and 1.5 U Taq DNA polymerase (TaKaRa, Japan). The reaction mixture was initially denatured for 3 min at 94 °C, then amplified for 30 cycles as follows: denaturation for 30 s at 94 °C, primer annealing for 30 s at 58 °C and extension for 30 s at 72 °C. A final extension step was done for 5 min at 72 °C (Thermocycler ABI 9700). An amplification product of 1000 bp by the outer primers was expected. In the second amplification step PCR was repeated as above with minor alterations: 1.0 µl of amplicon (the first step PCR products diluted 100-fold) was used for the second amplification step, primer annealing for 50 s at 58 °C, and inner-F (5' CTGGCG GCGTGCCTAA TAC 3')/inner-R (5' ACCCTC-TCAGGCC GGATACC 3') primers were used. The expected size of the amplicon was 250 bp or 430 bp. PCR product sequencing was performed by BGI Corporation with *helicobacter* (inner-F and inner-R) sequencing primers (Table 1). The sequences were determined by means of an automatic sequencer (3730XL; Applied Biosystems), the dye terminator reaction (Bigdye V3.1 Mix kit). For quality control, each DNA extraction and PCR amplification run included a positive control and negative control (double distilled water instead of sample). All PCR assays were performed in triplicate on three separate occasions.

2.5. Assay specificity

The specificity of the primers was tested under the above-mentioned conditions as well as with DNAs extracted from *C. jejuni*, *E. coli*, and *E. faecalis*.

Table 1 Comparison of the hemi-nested primers used in this study and other universal primers.

References	Sequence	Inclusive ^a	Evaluation ^b
Avena et al. (2000)	Forward HS1: 5' AACGATGAAGCTTCTAGCTTGCTAG 3' Reverse HS2: 5' GTGCTTATTCGTTAGATACCGTCAT 3'	8	3'-end dimer between the primers
Coppola et al. (2002)	Forward 5' ACGATGAAGCTTCTAGCTTGCTAG 3' Reverse 5' TGCTTATTCGTTAGATACCGTCAT 3'	9	3'-end Forward Primer dimer
Huang et al. (2004)	Forward 5' GTCATGACGGGTATCC 3' Reverse 5' ACTTCACCCAGTCGCTG 3'	21	High difference between (F/R) product and primer melting temperatures
Fukuda et al. (2002)	Forward 5' GCTATGACGGGTATCC 3' Reverse 5' GATTTTACCCCTACACCA 3'	30	High difference between (F/R) product and primer melting temperatures
Nilsson et al. (2006)	Outer forward 5' CTATGACGGGTATCCGGC 3' Outer reverse 5' CTCACGACACGAGCTGAC 3' Inner forward 5' CTATGACGGGTATCCGGC 3' Inner reverse 5' TCGCCTTCGCAATGAGTATT 3'	31	High difference between (F/R) product and primer melting temperatures High difference between (U/L) product and oligo melting temperatures Terminal stability of the Forward Primer is too high Terminal stability of the Upper Oligo is too high
Bohr et al. (2004)	Forward 5_-GCTATGACGGGTATCC-3_ Reverse 5_-TCGCAATGAGTATTCCTCT-3	31	High difference between (F/R) product and primer melting temperatures
Rocha et al. (2005a)	Forward C96: 5' ACGGGTGAGTAACGCATAGG 3' Reverse R4-16SCHPEC: 5' CCTACGTTACCTT GTT ACGAC 3'	10	High difference between (F/R) product and primer melting temperatures
Fox et al. (1998)	Outer forward C97: 5' GCTATGACGGGTATCC3' Outer reverse C05: 5' ACTTCACCCAGTCGCTG 3' Inner forward C97: 5' GCTATGACGGGTATCC 3' Inner reverse C98: 5' GATTTTACCCCTACACCA 3'	25	High difference between (F/R) product and primer melting temperatures
Pellicano et al. (2004)	Outer forward 5' ATTAGTGGCGCACGGGTGAGTAA 3' Outer reverse 5' TTAGCATCCCGACTTAAGGC 3' Inner forward 5' GAACCTTACCTAGGCTTGACATTG 3' Inner reverse 5' GGTGAGTACAAGACCCGGGAA 3'	6	High difference between (F/R) product and primer melting temperatures High difference between (U/L) product and oligo melting temperatures 3'-end dimer between the Forward and Reverse primers 3'-end dimer between the Upper and Lower oligos
Goto et al. (2000)	Forward 5' CTA TGACGG GTA TCC GGC 3' Reverse 5' CTC ACGACA CGA GCT GAC 3'	31	High difference between (F/R) product and primer melting temperatures Terminal stability of the Forward Primer is too high
Riley et al. (1996)	Forward H276f: 5' CTATGACGGGTATCCGGC 3' Reverse H676: 5' ATTCCACCTACCTCTCCCA 3'	29	Terminal stability of the Upper Primer is too high Excessive difference between product and primer melting temperatures
This study	Outer forward 5' CTGGCGGCGTGCCTAATAC 3' Outer reverse 5' CTCACGACACGAGCTGAC 3' Inner forward 5' CTGGCGGCGTGCCTAATAC 3' Inner reverse 5' ACCCTCTCAGGCCGGATACC 3'	34	None

^a Numbers in the fourth column indicate the number of *helicobacters* totally matched with the primer.

^b The primers were analyzed by OLIGOU7.0 on the basis of *H. pylori* 16S rRNA.

2.6. Assay sensitivity

To investigate the sensitivity of the primers in the setting of abundant genomic DNA of enteric bacteria, the nested PCR was performed using DNA extracted from fecal samples of a healthy human uninfected with *H. pylori*, *H. hepaticus* and *H. bilis*. The fecal samples were spiked with serially diluted suspensions of *H. pylori*, *H. hepaticus* and *H. bilis*. In some experiments, 10-fold dilutions (10^1 , 10^2 , 10^3 , 10^4 , 10^5 , and 10^6 CFU) of a known stock concentration of three *helicobacters* suspended in PBS were spiked into 200 mg fecal samples before DNA extraction.

2.7. Animal infection model and sample collection

Specific pathogen-free BLAB/c mice were purchased at 6–7 weeks of age from Nanfang University. To determine whether BLAB/c mice were free of *H. hepaticus* and *H. bilis* or not, we used our assay and conventional method (Fox et al., 1998; Rocha et al., 2005a). For mouse inoculation, *H. hepaticus* and *H. bilis* were adjusted to 10^{7-9} CFU/200 μ l. Three mice were infected with 200 μ l of one of the cultures by intragastric gavage. Once infection was established (1 and 6 weeks after inoculation), and confirmed by fecal PCR, the mice were sacrificed. Feces, liver and cecum samples were collected to evaluate the nested-PCR assay (Jeong et al., 2011). All procedures were approved by the Institute Animal Care and Use Committee of Nanfang Medical University.

2.8. Ethical compliance

The procedure for obtaining specimens from humans in this study complied with those recommended by the Regional Ethical Committee on Human Experimentation of Liuzhou People's Hospital. Written informed consent was obtained from each subject.

2.9. Patient

A healthy human was considered to be uninfected by *H. pylori* if no *H. pylori* was detected in the immunochemical staining followed by gastroscopic biopsy specimens and rapid urease test was negative. To determine whether healthy human were free of *H. hepaticus* and *H. bilis* or not, we used our assay and conventional method. This study included 5 patients with *H. pylori* infection and 5 healthy human without *H. pylori* infection. The feces samples from 5 patients with *H. pylori* infection and 5 healthy human regularly examined in a hospital in Liuzhou City were collected in September 2014. Samples were kept on ice during transfer to the Department of Microbiology, Liuzhou People's Hospital, and were tested as soon as possible. Neither patient nor healthy human has used following medications: H2 histamine blockers, antimicrobials, proton pump inhibitors and bismuth salts within the previous four weeks before samples were collected.

3. Results

The nested PCR primers were designed to the target 16S rRNA gene specific to all 34 type strains of *Helicobacter*

spp., not including any rare bases. Theoretically, the primers, being able to detect all the type strains, have not any faults according to the Oligo7.0 Software evaluation. In comparison with it, not only the inclusivity but also some faults lies in other primers usually used in the literature, as described in Table 1. Furthermore, the expected products size and optimal annealing temperature to 34 type strains were described in Table 2. Numbers in the sixth column indicate the positions of the primers to different type strains. The optimal annealing temperature fluctuates from 55.3 °C to 57.8 °C for the inner primers, and from 56.5 °C to 57.9 °C for the outer primers (Table 2). In practice, the excellent validity of the method was examined by feces simulated samples (Fig. 2), BLAB/c mice infection models (Fig. 3) and *H. pylori* infection fecal samples (Fig. 4).

DNAs extracted from *H. pylori*, *H. hepaticus* and *H. bilis* gave, respectively, a single amplification product of 997 bp, 990 bp and 1172 bp after the first-round PCR (Fig. 1).

Specificity of the genus-specific primer set was determined by amplifying DNAs prepared from the control strains. The expected DNA fragment was amplified from DNAs from the *Helicobacter* spp. tested (Fig. 2). Template DNAs from other bacterial genera commonly found in the human intestine were also evaluated for PCR amplification with the *Helicobacter* genus-specific primers. A product of the expected size was not observed with DNA from *E. coli*, *C. jejuni*, *E. faecalis*, *Bacteroides fragilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and negative control.

The clinical detection limits determined by spiking knowing amount of *H. pylori*, *H. hepaticus* and *H. bilis* into healthy human fecal sample, were all 10 CFU/100 μ l (Fig. 3), as previously described (Moyaert et al., 2008).

Nested PCR analysis was performed on liver, cecum and feces samples obtained from infected mice after intragastric administration of *H. hepaticus* and *H. bilis*. Nested PCR using DNA extracted from the feces of three mice before *H. hepaticus* infection produced no amplification signal whereas single amplification signal was observed using DNA extracted after *H. hepaticus* infection (Fig. 4A). DNA from liver and cecum samples from 3 *H. hepaticus* infection mice showed the 233-bp product except for one mouse' liver sample (Fig. 4B). DNA from feces samples collected before *H. bilis* infection did not show the 413-bp product with the nested PCR whereas the 413-bp product was observed after infection (Fig. 4C). DNA from liver and cecum samples from *H. bilis* infected mice all showed specific amplification with the nested PCR except for two liver samples (Fig. 4D).

4. Discussion

DNAs extracted from three feces samples from patients infected with *H. pylori* gave a single amplification product after our PCR but DNAs from two positive samples gave a single amplification product after C97/C05–C97/C98 PCR. Our PCR produced no amplification when DNAs obtained from feces samples of healthy human were used as template. Both C97/C98 PCR and C97/C05 PCR can't detect *H. pylori* in the positive fecal samples (Fig. 5B).

Molecular diagnostic methods using the polymerase chain reaction (PCR) are the gold standard in *Helicobacter* diagnostics. Most methods rely on the amplification of a section of the

Table 2 The location and optimal annealing temperature of the primers in 34 type strains of *Helicobacter* spp.^a

Taxon	Type strain	16S rRNA accession NO.	Location	Optimal annealing temperature ^b (°C)
<i>Helicobacter acinonychis</i>	<i>H. strain</i> 90-119/ATCC51101	M88148	36/270/1031	57.5/57.1
<i>Helicobacter anseris</i>	<i>H. strain</i> MIT 04-9362/ATCC BAA-1299	DQ415545	28/261/1020	57.5/57.4
<i>Helicobacter aurati</i>	<i>H. strain</i> MIT 97-5075c/ATCC BAA-1	AF297868	28/261-1017	56.7/57.6
<i>Helicobacter baculiformis</i>	<i>H. strain</i> M50 CCUG 53816/LMG 23839	EF070342	28/259/1010	57.1/57.9
<i>Helicobacter bilis</i>	<i>H. strain</i> Hb1/ATCC 51630	U18766	28/441/1199	56.5/57.2
<i>Helicobacter bizzozeronii</i>	<i>H. strain</i> Hänninen 1/Storkis/CCUG 35545	Y09404	9/240/1000	56.9/57.8
<i>Helicobacter brantae</i>	<i>H. strain</i> MIT 04-9366/ATCC BAA-1298	DQ415546	28/263/1021	57.4/57.7
<i>Helicobacter canadensis</i>	<i>H. strain</i> NLEP-16143/ATCC 700968	AF262037	13/246/1003	57.3/57.3
<i>Helicobacter canis</i>	<i>H. strain</i> ATCC 51401	AY631945	28/261/1020	57.2/57.6
<i>Helicobacter cetorum</i>	<i>H. strain</i> MIT 99-5656/ATCC BAA-540	AF292378	28/262/1023	56.9/57.6
<i>Helicobacter cholecystus</i>	<i>H. strain</i> Hkb-1/ATCC 700242	AY686606	28/261/1019	57.5/57.9
<i>Helicobacter cinaedi</i>	<i>H. strain</i> Fennell 165/CCUG 18818	M88150	28/262/1020	57.4/57.7
<i>Helicobacter cynogastricus</i>	<i>H. strain</i> JKM4/LMG 23188	DQ004689	28/259/1024	57.0/57.7
<i>Helicobacter equorum</i>	<i>H. strain</i> EqF1/CCUG 52199	DQ307735	26/259/1017	57.2/57.7
<i>Helicobacter felis</i>	<i>H. strain</i> CS1/ATCC 49179	M57398	28/259/1020	57.2/57.8
<i>Helicobacter fennelliae</i>	<i>H. strain</i> Fennell 231/ATCC 35684	M88154	36/622/1038	55.3/56.5
<i>Helicobacter ganmani</i>	<i>H. strain</i> CMRI H02/CCUG 43526/CIP 106846	AF000221	9/240/998	56.8/57.4
<i>Helicobacter heilmannii</i>	Strain ASB1 = DSM 24751	HM625820	36/267/1028	57.0/57.8
<i>Helicobacter hepaticus</i>	<i>H. strain</i> Hh-2/ATCC 51448	U07574	28/261/1017	57.3/57.7
<i>Helicobacter macacae</i>	MIT 99-5501 = LMG 26486	AF333338	72/470/1227	56.0/56.9
<i>Helicobacter marmotae</i>	<i>H. strain</i> MIT 98-6070/ATCC BAA-546	AF333341	28/261/1018	57.2/57.5
<i>Helicobacter mastomyrinus</i>	<i>H. strain</i> MIT 97-5574/ATCC BAA-1046	AY742307	28/550/1307	56.2/57.0
<i>Helicobacter mesocricetorum</i>	<i>H. strain</i> MU 97-1514/ATCC 700932	AF072471	12/245/1003	56.7/57.3
<i>Helicobacter muridarum</i>	<i>H. strain</i> ST1/ATCC 49282	M80205	33/266/1024	57.3/57.6
<i>Helicobacter mustelae</i>	<i>H. strain</i> R85-13-6/ATCC 43772	M35048	36/269/1028	57.8/57.6
<i>Helicobacter nemestrinae</i>	<i>H. strain</i> T81213-NTB/ATCC 49396	X67854	36/266/1027	56.8/56.7
<i>Helicobacter pametensis</i>	<i>H. strain</i> Seymour B9/ATCC 51478	M88147	35/270/1028	57.5/57.7
<i>Helicobacter pullorum</i>	<i>H. strain</i> ATCC 51801/CCUG 33837	AY631956	29/262/1020	57.6/57.8
<i>Helicobacter pylori</i>	<i>H. strain</i> Royal Perth Hospital 13487/ATCC 43504	U01330	36/271/1032	57.1/57.6
<i>Helicobacter rodentium</i>	<i>H. strain</i> MIT 95-1707/ATCC 700285	U96296	13/244/1002	57.2/57.6
<i>Helicobacter salomonis</i>	<i>H. strain</i> Inkinen/CCUG 37845/CIP 105607	U89351	13/244/1005	57.1/57.8
<i>Helicobacter suis</i>	<i>H. strain</i> HS1/DSM 19735	EF204589	18/249/1010	56.5/57.7
<i>Helicobacter trogonum</i>	<i>H. strain</i> LRB 8581/ATCC 700114	U65103	13/246/1002	57.5/57.8
<i>Helicobacter typhlonius</i>	<i>H. strain</i> MIT 97-6810/ATCC BAA-367	AF127912	13/405/1161	55.8/57.1

^a The location and optimal annealing temperature for the hemi-nested primers were determined by OLIGOU7.0.

^b The former temperature is for the inner primers, and the latter is for outer primers.

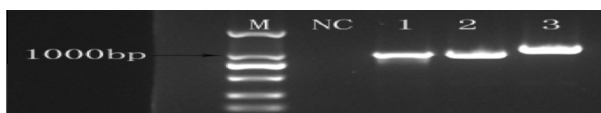


Figure 1 First PCR amplification of the 16S rRNA gene of *H. pylori*, *H. hepaticus* and *H. bilis*. Lane M, markers (Omega Marker DL-2000); NC, negative control; lane 1, *H. pylori*; lane 2, *H. hepaticus*; lane 3, *H. bilis*.

16S rRNA gene. Therefore, the validity and accuracy of results depend heavily on the PCR design and the time of its publication because new sequences are continually being submitted to

databases. Other target genes were also used as PCR target such as *cdtB*, *vacA*, *glmM*, *glm*, *cagA*, *rps4*, and 26 kDa's port.

Many studies in the past years have been always trying to find an excellent assay in inclusivity, sensitivity and specificity. The key to breakthrough is primers design. C97/C05–C97/C98 PCR cannot detect all positive *H. pylori* samples for primers itself. Another, multiple PCR is also very important. Regular PCR is difficult to detect helicobacter in fecal samples. Multiple PCR is more effective than regular PCR. In this report, we describe a hemi-nested PCR assay, which has excellent primers designed without any faults, for the detection of all species of *helicobacter* organisms formally named by LPSN. Actually, more faults lie in the primers designed for multiple PCR. In

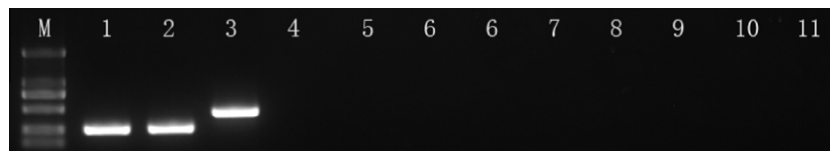


Figure 2 Ethidium bromide-stained agarose gel showing products resulting from amplification of DNA from various bacteria. Lane M, markers (Omega Marker DL-2000); lane 1, *H. pylori*; lane 2, *H. hepaticus*; lane 3, *H. bilis*; lane 4, *E. coli*; lane 5, *C. jejuni*; lane 6, *E. faecalis*; lane 7, *B. fragilis*; lane 8, *P. aeruginosa*; lane 9, *S. aureus*. Lane 10, negative control.

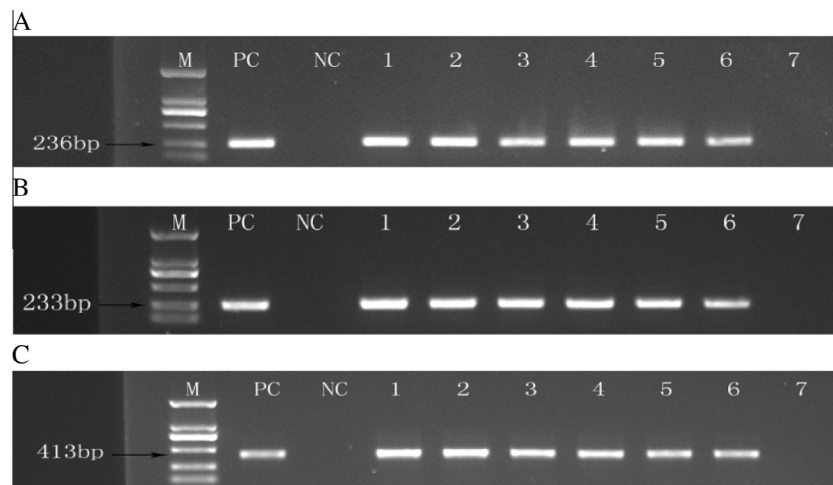


Figure 3 Sensitivity of detection of *H. pylori* (A), *H. hepaticus* (B) and *H. bilis* (C)-specific 16S rRNA gene by the nested PCR. Tenfold dilutions of pure bacterial culture were spiked into healthy human stool, and nested PCR was performed by using the extracted DNA from contaminate stool. Lane M, markers (Omega Marker DL-2000); PC, positive control; NC, negative control; lane 1, 10^6 CFU; lane 2, 10^5 CFU; lane 3, 10^4 CFU; lane 4, 10^3 CFU; lane 5, 10^2 CFU; lane 6, 10 CFU; lane 7, 1 CFU.

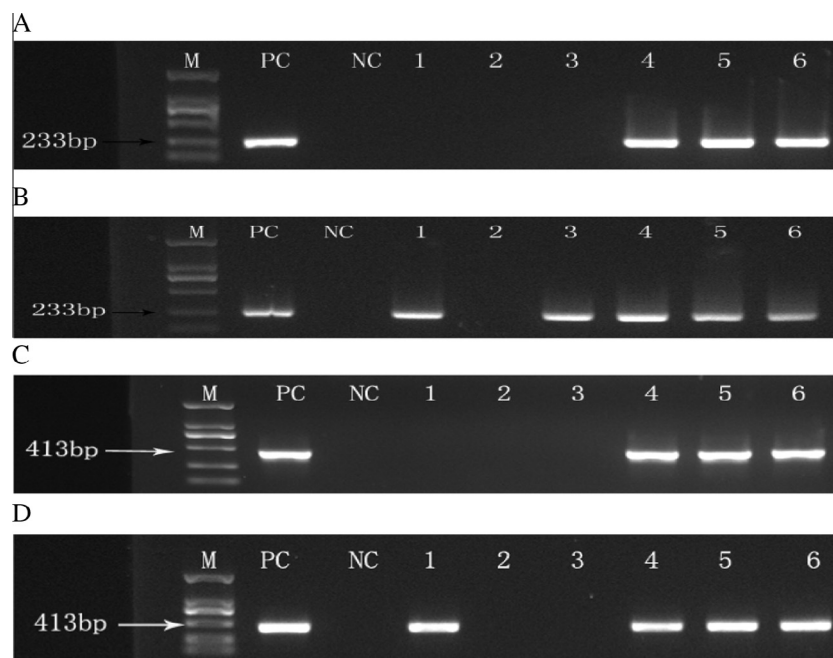


Figure 4 Nested PCR with experimental samples collected from *H. hepaticus* infected mice (A and B). Nested PCR amplification of DNA extracted from feces samples from 3 uninfected control (lane 1–3 in A) and 3 infected mice (lane 4–6 in A). Nested PCR amplification of DNA extracted from liver (lane 1–3 in B) and cecum samples (lane 4–6 in B) from 3 infected mice. Nested PCR with experimental samples collected from *H. bilis* infected mice (C and D). Nested PCR amplification of DNA extracted from feces samples from 3 uninfected control (lane 1–3 in C) and 3 infected mice (lane 4–6 in C). Nested PCR amplification of DNA extracted from liver (lane 1–3 in D) and cecum samples (lane 4–6 in D) from 3 infected mice. Lane M, markers (Omega Marker DL-2000); PC, positive control; NC, negative control.

view of it, our primers are more valuable. Besides, inspection of the 16S rRNA gene sequences from all 34 type strains revealed a 100% match with our *Helicobacter* genus-specific oligonucleotide primers. Thus, in retrospect, successful amplification of DNA from all *helicobacter* species DNA with these primers is expected.

It is known that PCR assays that function very specifically by using specific DNA samples as the template may not work properly when more complex DNA samples are studied (Bohr et al., 2002). We suspected that any fault of primers designed, even a base mismatch, may be amplified in the complex DNA samples. HS1/HS2 assay can detect more than 8 types of

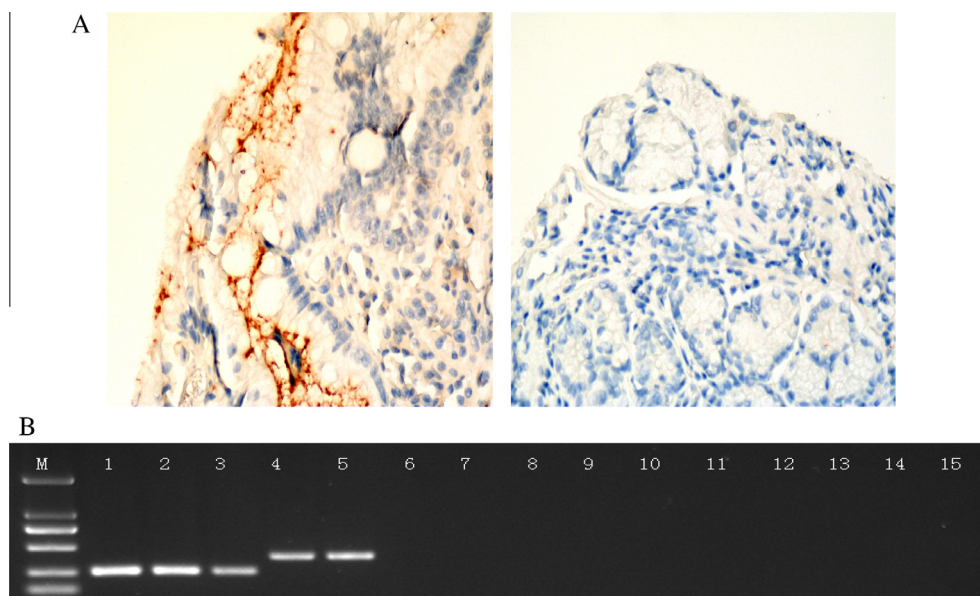


Figure 5 Nested PCR amplification of DNA extracted from feces samples from patients infected with *H. pylori* and uninfected control. (A) Left, gastric membrane from *H. pylori* infection patient. Right, gastric membrane from *H. pylori* non-infection patient (200x, Immunohistochemical staining). (B) Lane M, markers (Omega Marker DL-2000); lane 1–3, positive samples PCR using our primers; lane 4–6, positive samples PCR using C97/C05–C97/C98 primers; lane 7–9, negative samples PCR using our primers; lane 10–12, positive samples PCR using C97/C98 primers; lane 13–15, positive samples PCR using C97/C05 primers.

helicobacters using the purified DNA extracts as the template though it cannot totally match helicobacters 16S rRNA. Where applicable, the assay performed suboptimally on inclusivity. The investigators chose to include DNA extracts from other bacteria commonly found in the gastric and/or intestinal flora to evaluate the specificity of their assays. Moyaert et al. showed difficulties to accurately discriminate closely related taxa (Moyaert et al., 2008). Therefore, in our study, additional DNA extracts from feces of human uninfected with *H. pylori*, *H. hepaticus* and *H. bilis* were used to test assay specificity. No non-specific amplification was found. The assay sensitivity reached 10 bacterial cells per reaction mixture. The capability of the PCR assay was further tested by detection of *H. hepaticus* and *H. bilis* from samples obtained from infected mice, including liver, cecum and feces. Both bacteria were successfully identified. Moreover, we also evaluated the capability of the PCR assay by testing feces from patients infected with *H. pylori*. *H. pylori* was successfully identified in the feces of all infected patients. In this study, we found *H. pylori* test in fecal samples is more difficult than *H. hepaticus* and *H. bilis*. We guessed that fecal test is more sensitive for enterohepatic helicobacters than gastric helicobacters for the colonized location. Enterohepatic helicobacters DNA in fecal sample is more than gastric helicobacters. It was found that frozen fecal sample from *H. pylori* infection patients is apt to false negative results. We found that the *H. pylori* DNA in process of thawing may be partly destroyed. For more accurate results, we use the fresh fecal samples when testing *H. pylori*.

5. Conclusion

In our study, it was demonstrated that the hemi-nested PCR assay is highly reliable and sensitive for the genus-level identification of *Helicobacter* spp. This assay is able to detect all the

currently known species of helicobacter and identify different species of *helicobacter*. Therefore, it can serve as a powerful tool for the diagnosis of the *helicobacter* infections.

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