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An Alternative, Sensitive Method to Detect *Helicobacter pylori* DNA in Feces

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Keywords

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Abstract

Background: Despite the high sensitivity and specificity of PCR, detection of *Helicobacter pylori* DNA in feces is still challenging. Fecal samples contain inhibitory molecules that can prevent amplification of the target DNA. Even by using specific DNA extraction kits for stools, monitoring of infection by analyzing stool samples remains problematic and endorses the need for improved diagnostic methods.

Materials and Methods: The newly proposed method uses selective hybridization of target DNA with biotin-labeled probes, followed by DNA isolation with streptavidin-coated magnetic beads. After three washing steps, the purified DNA can be amplified immediately using conventional or quantitative PCR. In order to test this technique on biological samples, Mongolian gerbils were infected with *H. pylori* ATCC 43504 and fecal samples were analyzed on days 1, 4, and 10 post infection.

Results: A detection limit of one bacterial cell per 100 mg stool sample was established, but only after removal of the magnetic beads from the target DNA by heating. This resulted in a 10-fold increase of sensitivity compared to a commercially available stool DNA extraction kit. Analysis of fecal samples from infected gerbils demonstrated the presence of *H. pylori* DNA on each time point, while the uninfected animal remained negative.

Conclusions: The proposed technique allows detection of very low quantities of *H. pylori* DNA in biological samples. In laboratory animal models, detailed monitoring of infection and complete clearance of infection can be demonstrated thanks to the low detection limit.

Since its discovery by Marshall and Warren in 1983, the clinical importance of *Helicobacter pylori* infections has been widely recognized [1]. In 1994, *H. pylori* was classified as a group I carcinogen by WHO since it increases the incidence of malignant neoplasms, gastric cancer, and MALT lymphoma in particular [2]. As a result of these severe complications, the European Helicobacter Study Group (EHSG) recommended in 2007 to eradicate the bacterium in patients with different types of gastro-duodenal disease [3]. The first-choice treatment consists of triple therapy using a proton-pomp inhibitor with clarithromycin, and amoxicillin or metronidazole given twice daily [3]. However, the widespread use of these antibiotics has led to the development of drug-resistance [4]. Continued in vitro and in vivo research on novel treatment strategies is therefore pivotal. For this purpose, animal models of *H. pylori* in rats, Balb/c mice and gerbils have been widely used to evaluate anti-*Helicobacter* compounds and as suitable laboratory models to study gastric carcinogenesis [5,6]. Currently, infection rates can only be estimated by assessing the amount of bacteria in the stomach at necropsy, hence requiring a high number of animals. For example, studying the efficacy of a novel antibacterial agent over a period of time requires sacrifice of several animals at each studied time point. For these reasons, it would be very advantageous to dispose of a highly sensitive non-invasive technique that allows monitoring of *H. pylori* infection rates in laboratory animals for research and which could also be used in patients for clinical diagnosis.

In the present study, a highly sensitive and specific method to extract *H. pylori* DNA from fecal samples was

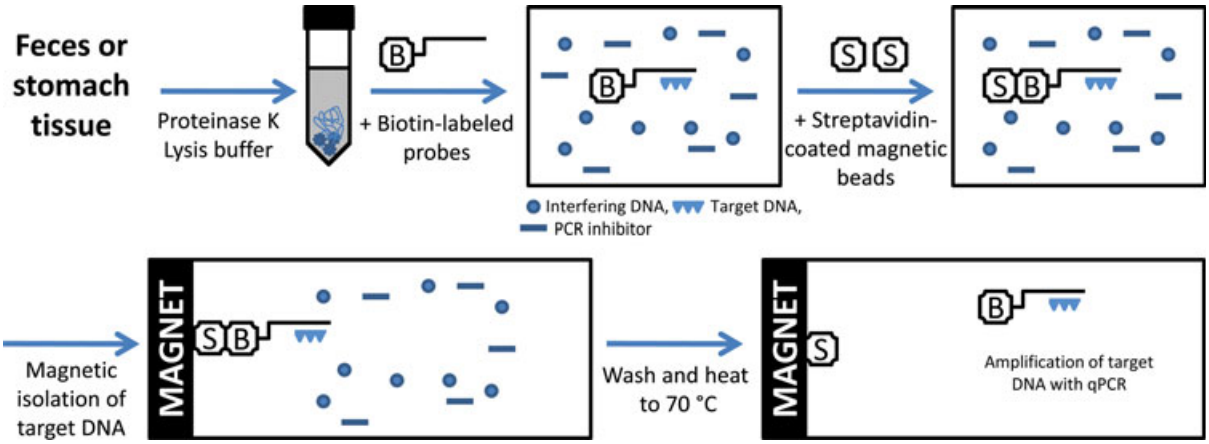

Figure 1 Schematic representation of the selective hybridization technique. DNA is liberated from biological samples by treatment with proteinase K and lysis buffer. Biotin-labeled probes are added, which will hybridize with *Helicobacter pylori* DNA in the sample. After addition of streptavidin-coated magnetic beads, *H. pylori* DNA is selectively isolated by placing samples in a magnetic block. Interfering DNA and PCR inhibitors are removed by several washing steps, allowing immediate amplification of purified DNA.

developed that should allow to monitor the *H. pylori* infection rate over a period of time in the same animal. The pathogenesis of *H. pylori* infection in Mongolian gerbils (*Meriones unguiculates*) has the advantage of being quite similar to the disease development in humans and was therefore selected as animal model in this study [7]. The proposed technique, schematically represented in Fig. 1, uses selective hybridization of target DNA with biotin-labeled probes, followed by extraction of DNA with streptavidin-coated magnetic beads. After several washing steps, the purified *H. pylori* DNA can be amplified using conventional or qPCR. This method was published for the first time in 1997 by Mangiapan et al. [8] for the detection of *Mycobacterium tuberculosis* in paucibacillary clinical sputum samples. In analogy, the amounts of *H. pylori* DNA in fecal samples are also very low. In addition, it is well-known that the presence of interfering DNA and remnants of PCR-inhibitors, such as polysaccharides and excess proteins, may hinder detection of *H. pylori* with commercially available DNA extraction kits. Introduction of selective capture of target DNA and several washing steps improves selective hybridization, resulting in highly sensitive and more specific detection of *H. pylori*.

Materials and Methods

Animals and *Helicobacter pylori* Strain

Female, specific pathogen-free Mongolian gerbils (Janvier, Le Genest-St-Isle, France) were maintained at 20 °C, 50% humidity and at a light/dark cycle of 12/12 hours. They had free access to standard rodent food pellets (Carfil Quality, Turnhout, Belgium) and tap water. All experiments and procedures were approved by the ethical committee of the University of Antwerp.

Helicobacter pylori strain 43504 was purchased from the American Type Culture Collection (LGC Standards S.a.r.l., Molsheim, France). The strain has a functional *cag* pathogenicity island, VacA s1m1 and functional BabA and OipA [9]. It has been demonstrated that it colonizes gerbils consistently for at least 1 year and causes reproducible mucosal damage [9]. The strain was grown on Tryptic Soy Agar (TSA) (Lab M Limited, Lancashire, UK) plates, supplemented with 5% sheep blood (Oxoid, Cambridge, UK) at 37 °C under micro-aerophilic conditions (5% O_2, 10% CO_2, 85% N_2), generated by a Whitley H35 Hypoxystation (Don Whitley, West Yorkshire, UK). To inactivate *H. pylori*, bacteria were placed at 24 °C for 72 hours in a normoxic environment. Subsequently, inactivation was checked by culture and it was tested whether the inactivated bacteria could still be detected by PCR using 16s RNA primers (see below).

Infection of Mongolian Gerbils with *Helicobacter pylori*

Mongolian gerbils (n = 3, age = 5 weeks, ♀) were fed 0.8 mL of Tryptic Soy Broth (TSB) (Lab M Limited, Lancashire, UK) containing 1×10^9 CFU/mL using feeding needle, three times with 48-hours intervals. The gerbils were fasted 24 hours before each inoculation until 4 hours after gavage. One gerbil was given TSB alone and served as negative or uninfected control.

In addition, two groups of three Mongolian gerbils were infected with either viable (7.7×10^7 CFU) or

inactivated (approximately 10^8 CFU) *H. pylori*. Excretion of *H. pylori* DNA in fecal samples was monitored using the method described above for 4 days after infection. After 4 days, the animals were killed using CO_2 overdose. The stomachs were removed aseptically, 100 mg samples were put in 550 μL lysis buffer and 55 μL proteinase K and analyzed as described below for fecal samples.

Spiking of Fecal Samples

Helicobacter pylori negative fecal samples were collected from uninfected gerbils. These samples were spiked after homogenization with a known amount of bacterial cell suspension ranging from 1 to 10^4 CFU/100 mg feces.

Homogenization and Digestion

For each bacterial concentration, 100 mg of feces (corresponding to 2–5 pellets, depending on the size) was collected and dissolved in 1 mL 0.2% Tween 80 (Sigma-Aldrich, Bornem, Belgium) solution. The samples were vortexed vigorously until all fecal pellets were homogeneously suspended. After 10 minutes of sedimentation, the supernatant was transferred to a new 1.5 mL tube and centrifuged, first at 3800 g for 1 minute. The supernatant was transferred to a new 1.5 mL tube and centrifuged at 20800 g for 5 minutes. The pellet was re-suspended in 550 μL lysis buffer (100 mmol/L Tris–HCl pH 7.4, 150 mmol/L NaCl, 50 mmol/L EDTA all purchased from Sigma-Aldrich, Bornem, Belgium) and 55 μL proteinase K (20 mg/mL, Ambion®; Invitrogen, Merelbeke, Belgium) was added for digestion overnight while shaking at 50 °C. Samples were stored at −20 °C until further processing.

Selective Hybridization and DNA Elution

The digested samples were heated at 100 °C for 10 minutes and then cooled to 0 °C on ice. After adding 200 μL NaCl 3.75 mol/L solution, containing 3.3 pmol of each biotin probe (developed using Amplify-X 3.1.4) (Integrated DNA technologies, Leuven, (Belgium)) (Table 1), samples were incubated for 2.5 hours at 60 °C while shaking, cooled to room temperature and 10 μL of Dynabeads® M-280 Streptavidin (Dynal, Invitrogen, Merelbeke, Belgium) was added. The beads had been washed according to the manufacturer's instructions. Subsequently, tubes were incubated at room temperature for 2 hours to allow streptavidin–biotin complexation. The beads were next concentrated by placing samples in a magnetic block for 1 minutes (Dynamag™-2 magnet, Dynal, Invitrogen, Merelbeke, Belgium), washed twice with Tris–HCl (10 mmol/L, pH 7.5), containing 0.1 mmol/L EDTA and once in 20 μL Tris–HCl solution (10 mmol/L, pH 8), containing 1 mmol/L EDTA. To improve PCR amplification, the captured DNA was released from the beads by heating the samples to 70 °C [10]. The magnetic beads and the eluted DNA were separated using the magnetic block and 5 μL of this solution was used for amplification.

Table 1 Sequences of oligonucleotide primers [11] and probes for *Helicobacter pylori* 16S rRNA

Forward primer	5′ TCG GAA GTG GAG CCA ATC TT 3′
Reverse primer	5′ - GGA ACG TAT TCA CCG CAA CA - 3′
Forward probe	5′ biotin - TEG - GTG CTA CAA TGG GGT GCA CAA AGA 3′
Reverse probe	5′ biotin - TEG - TGA TCC AAC CGC AGG TGT CAC TA 3′

Analysis of Human Samples

A stool sample (quintuplicate) of a confirmed *H. pylori* positive patient was analyzed as described above. Presence of the bacterium in the stomach of the patient (34-year old male,) was demonstrated after gastroscopy by rapid urease test (BioChimika, Fluka, Buchs, Switzerland) and culture [11]. As negative control, a stool sample of a healthy, female (26 years old) volunteer was analyzed in parallel.

Stool DNA Extraction Kit

The QIAamp DNA Stool Mini kit (QIAgen, Venlo, the Netherlands) was applied according to the manufacturer's instructions. The DNA was eluted in 200 μL AE buffer and 1 μL of this solution was used for amplification.

Real-time PCR Assay

The PCR-reactions were performed according to the protocol described by Yamazaki et al. [12] in which 16s RNA primers were used because of their constitutive nature and 100% specificity. Real-time PCR (StepOnePlus; Applied Biosystems, Foster City, California, USA) was performed in a 20 μL reaction mixture, containing 10 μL of ''Power SYBR® Green PCR Master Mix'' (Applied Biosystems, Foster City, California, USA), 4 pmol of each primer, 5 μL of each DNA sample or a 10-fold serially diluted standard of *H. pylori* DNA (11.99–11.99×10^5 fg/μL) and water for molecular biology (Sigma-Aldrich, Bornem, Belgium) in 96-well optical plates (Applied Biosystems, Foster City, California,

USA). The PCR protocol was conducted using an initial denaturation step at 95 °C for 10 minutes and 40 cycles at 95 °C for 15 seconds and 60 °C for 1 minute.

Results

PCR Assay

In three independent spiking experiments, the recovery of added bacterial DNA was about 1–5%. To increase the PCR efficiency, the interference of the magnetic beads in the PCR mixture was evaluated. Their impact on the fluorescent SYBR® Green signal could clearly be demonstrated, since the amplification plot in the presence of beads propagated differently than when the beads were removed (Fig. 2). In the presence of beads, the C_T value increased, the slope of the linear phase was less steep and the saturation phase was reached at a lower fluorescent intensity. In the next experiments the captured DNA was released from the beads by heating the samples to 70 °C, leading to a recovery of added bacterial DNA of about 90–110%.

Comparison of Selective Hybridization Technique with Stool DNA Extraction Kit

To compare the performance of the proposed selective hybridization technique with a commercially available stool DNA extraction kit, three independent spiking experiments were performed. A 10-fold dilution series of *H. pylori* was added to 100 mg samples of uninfected gerbil feces. Tests were performed on three different days in triplicate for both methods and the recovered amounts of *H. pylori* DNA were determined using qPCR. The final elution volumes differ between both methods, therefore a multiplication factor of 50 was implemented to compensate for the dilution effect of the extracted DNA in the commercial kit. The selective hybridization technique allows a more sensitive detection of *H. pylori* compared to samples analyzed with the QIAamp DNA Stool Mini Kit, which failed to detect the lowest concentration of added CFU's (Fig. 3).

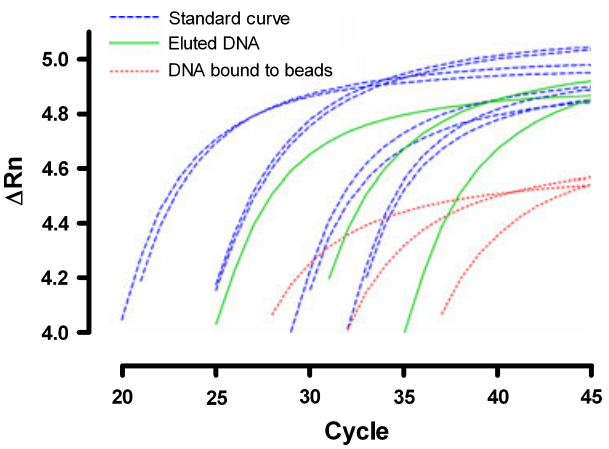

Figure 2 Amplification plot of purified *Helicobacter pylori* DNA, either with or without Dynal® beads in the PCR mixture. (representative example of three independent experiments) DNA which has been released from the beads is detected earlier and has a clear-cut increase in fluorescent signal. When Dynal® beads are present in the mixture, the signal appears much later and is lower.

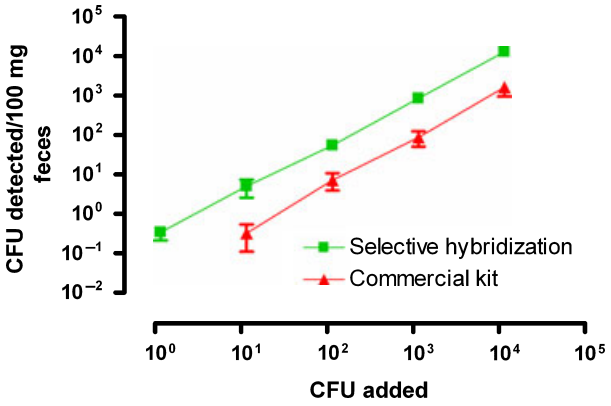

Figure 3 Comparison of CFU detected in fecal samples spiked with *Helicobacter pylori* (1.15–1.15 × 10^4 CFU/100 mg feces). The selective hybridization technique (■) allows more sensitive detection of *H. pylori* DNA compared to samples analyzed with the QIAamp DNA Stool Mini kit (▲), as the lowest concentration cannot be detected by the kit. The amounts of CFU detected differ significantly between both methods (n = 3, p = .02, Two-way ANOVA).

Analysis of Samples from *Helicobacter pylori* Infected Gerbils

Gerbils (n = 3) were infected with *H. pylori* ATCC 43504 three times with 48 hour intervals. One gerbil was sham-infected with TSB alone and served as negative control. On days 1, 4, and 10 after infection, fecal samples were collected and analyzed using the selective hybridization technique. The extracted amounts of DNA were converted to log-values. On day 1, the amount of recovered DNA was high and must be related to the excretion of the infection inoculum. On days 4, and 10, the number of bacteria excreted decreased to lower, stable levels (Fig. 4).

To discriminate between a positive PCR signal originating from the inoculated bacteria and bacteria that actually colonized the stomach, it was assessed for how long the inoculated bacteria contributed to the PCR signal. In a first set up, gerbils (n = 3) were infected once with a high amount of inactivated *H. pylori* ATCC 43504. The presence of *H. pylori* DNA in stool samples was

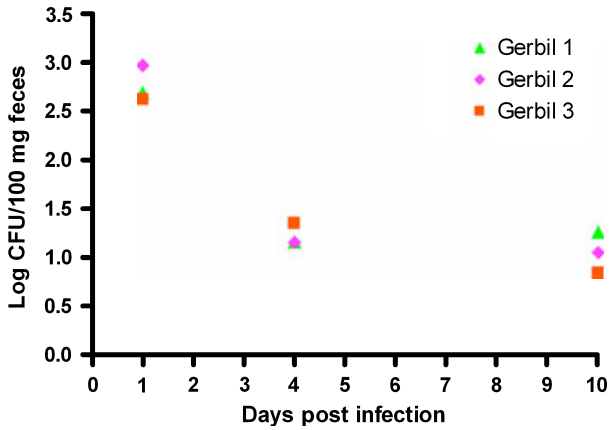

Figure 4 Monitoring of the amount of *Helicobacter pylori* present in stool of Mongolian gerbils up to 10 days post infection (DPI) using the selective hybridization technique. On day one after infection, the number of bacteria detected is higher than on days 4 and 10. This is due to the excretion of excess bacteria coming from the inoculum. On the three time points analyzed, the sham-infected animal was negative.

monitored for 4 days. Twenty-four hours after infection, no *H. pylori* DNA was found in any of the specimens.

In addition, a second group of gerbils (n = 3) was infected only once with viable *H. pylori*. Stool samples were analyzed up to 4 days after infection. Whereas, a weak PCR signal was detected after 24 hours (0.041 ± 0.020 CFU/100 mg (mean ± SEM)), all samples became negative after 48 hours. Four days after inoculation, animals were sacrificed and 16s RNA PCR analysis of the stomach showed to be negative. This demonstrates that when infection is not successful, negative results will be obtained in both stomach and fecal specimens within 2 days after inoculation.

Analysis of Human Samples

Samples were analyzed in quintuplicate and amounts of 0.107 ± 0.048 (mean ± SEM) CFU/100 mg feces were detected. The negative control samples were negative.

Discussion

The objective of this study was to develop a more sensitive and specific technique to monitor *H. pylori* infections in laboratory animals by analyzing fecal samples. Generally used techniques to extract DNA from feces, such as phenol–chloroform–isoamyl alcohol extraction and QIAamp DNA Stool Mini kit (QIAgen, Venlo, the Netherlands), yield DNA templates which contain PCR inhibitors and have high complexity [13]. Also due to the presence of different targets, sensitivity is limited to approximately 60–90% compared to standard diagnostic techniques [14–17]. To monitor *H. pylori* infections in stools, sensitivity should be increased while still maintaining specificity [12]. Using the custom-made, biotin-labeled *H. pylori* DNA probes of the selective hybridization technique as adapted from Mangiapan et al. [8], only *H. pylori* DNA is extracted from feces. PCR inhibitors and interfering DNA templates can be removed easily by three washing steps. As such, full recovery could be established.

The method developed by Mangiapan et al. [8] was adopted with minor modifications (Fig. 1). Since previous studies either used normal, non-quantitative PCR or did not compare the amount of DNA extracted with the amount of bacteria added, the influence of the magnetic beads on the fluorescent SYBR® Green signal was not determined [18,19]. While performing some quantitative experiments, it was noted that the retrieval of bacterial DNA was incomplete. When DNA amplification was performed in the presence of beads in the PCR mixture, only about 1–5% of the added DNA was recovered. After separation of beads and captured DNA using the method described by Holmberg et al. [10], the recovery increased to values between 90 and 110% (Fig. 2) [10]. This observation demonstrates that the beads indeed interfere with the quantitative PCR technique.

To determine whether the strategy of selective extraction of *H. pylori* DNA from fecal samples actually leads to an increased sensitivity, the performance of the selective hybridization technique was compared with the commercially available QIAamp DNA Stool Mini Kit in three independent experiments on 100 mg feces samples spiked with a 10-fold dilution series of *H. pylori* bacteria. The samples were analyzed in triplicate and the amounts of extracted DNA were determined using qPCR (Fig. 3). It is clearly shown that selective hybridization allows more sensitive detection of *H. pylori* DNA, illustrated by the fact that the lowest concentration of 1 CFU/100 mg feces cannot be detected by the kit. Moreover, the selective hybridization technique showed to be more sensitive than the QIAgen kit at each dilution tested. Even though the detection limit of 10 CFU/100 mg feces may seem satisfactory, it is only sufficient for samples which contain large amounts of DNA, e.g. gut microbiota [20]. The monitoring of *H. pylori* DNA in fecal samples requires a more sensitive technique, one that can easily quantify *H. pylori* infection rates. In order to test the selective hybridization technique on actual biological samples, three gerbils were infected with *H. pylori* ATCC 43504 and fecal samples were analyzed on day 1, 4, and 10 after infection. Presence of *H. pylori* DNA could be demonstrated in each sample of every animal, while the uninfected control maintained a negative status.

Since the technique proposed in this article cannot distinguish between inoculated *H. pylori* and colonized

H. pylori in the stomach, additional experiments were performed to determine at which moment after infection the test can be performed. Two groups of gerbils were infected with either viable or inactivated *H. pylori* and excretion of DNA was monitored. After only 24 hours the stool samples of the inactivated *H. pylori* group were negative. This means that after only two transit cycles, all DNA has been cleared from the gut and therefore confirmation of infection can be performed 24 hours after gavage. Stool samples of gerbils infected only once with viable *H. pylori* were found negative when analyzed 48 hours after inoculation. The difference between active and inactivated *H. pylori* can probably be explained by adherence of the active bacteria to the stomach wall. It shows that bacteria that can adhere to the stomach wall, but not successfully colonize it, are cleared from the animal in <48 hours. These data confirm the specific nature of the method, meaning that when infection is not successful, stomach, and stool samples will also give a negative result only 2 days after inoculation.

To demonstrate the reproducibility of the method, a stool sample of a confirmed *H. pylori* positive patient was examined according to the described protocol. Even though the amounts of DNA recovered from the sample were low, these data confirm the usefulness of the method regarding the analysis of clinical samples.

In conclusion, the newly developed method allows the monitoring of *H. pylori* infections in gerbils by analyzing feces without having to sacrifice them. Hence, this technique will be a valuable tool to monitor the progress of *H. pylori* infections both in laboratory animals and in human patients. Further studies are now ongoing to optimize and to evaluate the use of this technique for diagnosis of *H. pylori* infections in clinical settings.

Acknowledgements and Disclosures

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