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Original Article

Evaluation of a Specific Modulation Frequency Technique for Detecting *H. pylori* in a Model of Experimentally Infected Mice

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Abstract

Diagnosis of *Helicobacter pylori* is challenging. Diverse techniques have been developed for accurate diagnosis. However, they have some limitations. This research investigated the efficacy of specific modulation frequency (SMF) compared to other routine diagnostic methods for detecting *H. pylori* in gastric biopsy samples of experimentally infected mice. One-hundred and fifty healthy male C57BL/6 mice were included and divided into the control and treatment groups. The mice in the treatment group were treated with 0.2 ml of 0.2 M NaHCO3 to neutralize gastric acidity. Then, 109 colony-forming units of *H. pylori* (ATCC 43504) mixed in PBS were used intragastrically to inoculate the mice. Mice were kept for up to 28 days and examined on days 0, 7, 14, 21, and 28 using culture, polymerase chain reaction (PCR), and SMF. On day 0, only the SMF and PCR could detect the *H. pylori* in the stomach of 60% and 20% of mice, respectively. On day 7, culture, PCR, and SMF could detect *H. pylori* in 40%, 80%, and 100% of mice, respectively. SMF detected all infected mice from days 7 to 28 (100%). PCR detected all *H. pylori*-infected mice at days 14 to 27 (100%). Another test (culture) detected all infected mice only on day 28. Significant differences were found among the three diagnostic methods on days 0, 7, 14, and 21 of the experiment (P<0.05). SMF was found to have high sensitivity and specificity for *H. pylori* detection in the early stages of infection.

Keywords: *Helicobacter pylori*, Specific modulation frequency, Diagnosis, Mice

1. Introduction

As a gram-negative, microaerophilic flagellated bacterium, *Helicobacter pylori (H. pylori)* is responsible for a wide range of conditions, including gastric adenocarcinoma, peptic ulcer disease, type B gastritis, duodenal ulcer, mucosa-associated lymphoid tissue (MALT) lymphoma, and gastric B-cell lymphoma (1). The main reservoir of *H. pylori* in the human stomach (2). Additionally, the main route of infection is fecal-oral and oral-oral (2). *H. pylori* resistance to antibiotics has become an emerging issue (3). Documented surveys have shown that *H. pylori* isolated from clinically infected humans are highly resistant to various antibiotics, including penicillins, macrolides, aminoglycosides, fluoroquinolone, tetracyclines, and sulfonamides (2).

According to the primary evidence, infection of *H. pylori* is very common and causes a variety of medical challenges for health establishments; thus, health management of *H. pylori* infection is essential. *H. pylori*-induced infections are highly prevalent in both developed and under-development states (4). Although

the medical community has attempted to treat and vaccinate *H. pylori* simultaneously, selective treatment and accurate screening of the target population remains a major challenge for health organizations. Additionally, many vaccines have been developed to prevent the disease, but none are licensed for use by relevant agencies (5). Thus, rapid diagnosis of *H. pylori* infections is the practical approach to preventing the disease spread in the human population.

Several diagnostic techniques for clinical detection of *H. pylori*, particularly gastric biopsy. Microbial culture is regarded as the gold standard for detecting *H. pylori*. However, culture is time-consuming and needs microaerophilic conditions for *H. pylori* isolation (6). Several PCR-based molecular techniques have also been developed to detect *H. pylori* DNA. However, they cannot differentiate between dead or alive bacteria and only detect DNA sequences (7). Several clinical studies show deficient and safe levels of amplitude-modulated electromagnetic fields applied by an intrabuccal spoon-shaped probe could provoke diagnostic and remedial properties (8). Although the positive effects of various electric and electromagnetic fields (EMFs) on medical sciences are apparent (9, 10), the application of this technology has been limited to specific fields, maybe, due to the poor understanding of its mode of action. EMFs have the potential as diagnostic tools for the future of non-invasive medical diagnosis and treatment.

This study aimed to assess the potential of the specific modulation frequency (SMF) technique compared with the microbial culture and PCR in detecting *H. pylori* in mice.

2. Materials and Methods

2.1. Animal Models

A number of 150 healthy male C57BL/6 mice (aged between 6-8 weeks) were purchased from Pasteur Institute, Iran. All purchased mice were healthy and maintained in static micro isolator cages. All mice were placed in a pathogen-free environment with a 12:12-h light-dark and constant room temperature. The mice had free access to food pellets and water. Animal welfare requirements were rigorously followed during the experiments following the Iranian Society's recommendations for the Prevention of Cruelty to Animals and Islamic Azad University Research Council (IR.IAU.SRB.REC.1398.186)

2.2. Bacterial Strains

Helicobacter pylori (ATCC 43504) was purchased from the Razi Institute (Karaj, Iran). The traditional culture was grown on Blood Agar Base (Sigma, St. Louis, MO, USA) containing 5% horse serum, trimethoprim (30 mg/L) (both Sigma, St. Louis, MO, USA), cycloheximide (100 $mg.L^{-1}$), vancomycin (10 $mg.L^{-1}$), and nalidixic acid (30 $mg.L^{-1}$). MART system (Lichtenvoorde, Netherlands) was used to achieve microaerophilic conditions (85% nitrogen, 10% CO^2, and 5% oxygen). The culture medium was incubated at 37 °C for 2 days. A Brucella broth (Merck, Germany) medium supplemented with 20% glycerol and 25% fetal calf serum (Gibco, USA) was prepared and used to store the single colony strains of *H. pylori* at -80 ˚C for further analysis.

2.3. Treatments and Mice Inoculation

After being in quarantine for a week, the mice were assigned into two groups treatment and control. The mice in the control group (75 mice) were kept under the standard conditions without any inoculation with bacteria. The treatment group (75 mice) was inoculated with *H. pylori*. The strains of *H. pylori* were first cultured in a microaerophilic medium, harvested in sterile phosphate-buffered saline (PBS) with a pH of 7.4, and blended with identical density. Mice of the treatment group were fasted overnight but provided free access to water. The mice were treated with 0.2 ml of 0.2 M NaHCO3 (Merck, Germany) to neutralize gastric acidity. Then, 109 colony-forming units of *H. pylori* (ATCC 43504) mixture of PBS were used intragastrically to inoculate the mice. The procedure was replicated 2 or more times at 2-day intervals. Mice were kept for up to 28 days. *H. pylori* strains were diagnosed on days 0, 7, 14, 21, and 28 of the experiment using different methods. For each

diagnostic method, 5 mice were selected each day of the experiment (15 mice each day).

2.4. Culture-Based Identification of *H. pylori*

Gastric mucosal biopsy samples were obtained from all parts of the stomach. The gastric sample of each mouse was separately inoculated on Brain Heart Infusion (BHI, Merck, Germany) media and homogenized and cultured in brucella blood agar (Merck, Germany) supplemented with trimethoprim (30 $mg.L^{-1}$), ferrous sulfate, sodium pyruvate (all from Sigma, St. Louis, MO, USA), cycloheximide (100 mg/L), vancomycin (10 $mg.L^{-1}$), and nalidixic acid (30 $mg.L^{-1}$). Media were cultured in a microaerophilic situation (85% nitrogen, 10% CO^2, and 5% oxygen) using the MART system (Lichtenvoorde, The Netherland) for 48-72 h at 37°C. In order to identify the suspicious colonies, the morphology of the colony was examined, and specific biochemical tests (urease, oxidase, and catalase tests) were performed.

2.5. PCR-Based Identification of *H. pylori*

A DNA extraction kit (Thermo Fisher Scientific, St. Leon-Rot, Germany) was used to extract the genomic DNA from the gastric biopsy samples. The procedure was performed in accordance with the guidelines provided by the kit manufacturer. Then, the quality and purity (A260:A280) of the extracted DNA were inspected using electrophoresis and NanoDrop (Thermo Scientific, Waltham, MA, USA). Moreover, a PCR thermal cycler (Eppendorf Co., Hamburg, Germany) was used to perform PCR in accordance with the described procedure (11). The primers and PCR conditions used in detecting *the 16S rRNA* gene of *H. pylori* are listed in table 1. *H. pylori (26695) strain* and sterile PCR grade water (Thermo Fisher Scientific, Germany) were used as the positive and negative control, respectively.

2.6. Specific Modulation Frequencies (SMF)

Finally, the anesthesia was induced using ketamine (100 $mg.kg^{-1}$) and resolved after stomach sample collection. Then the samples were assessed using the SMF according to the company's instructions (MINI-EXPERT-DT, Russia). The current measurement of the device was 6 µA @ 100 kOhm. The device determined the polarity of electro-therapy as positive, negative, and bipolar pulses. For the *H. pylori* detection, pulse amplitude and pulse frequency were adjusted on 12 V and 1-100 Hz, respectively. IMIDIS-EXPERT software (IMIDIS-EXPERT, Russia) was used for data analysis.

2.7. Data Analysis

Data were analyzed in IBM SPSS Statistics V21.0 software. The relationships between the variables were investigated using Chi-square and Fisher's exact two-tailed tests. The level of significance was considered as $P<0.05$.

Table 1. List of primers and PCR conditions used to detect *H. pylori* in gastric biopsy samples

Genes	Primer Sequence (5'-3')	Size of product (bp)	Volume of PCR reaction (50 µl)	PCR programs
16SrRNA	F: CTGGAGAGACTAAGCCCTCC R: ATTACTGACGCTGATTGTGC	110	5 µL PCR buffer 10X 2 mM $Mgcl_2$ 150 µM dNTP (Thermo Fisher Scientific, St. Leon-Rot, Germany) 0.75 µM of each primer F & R 1.5 U Taq DNA polymerase (Thermo Fisher Scientific, St. Leon-Rot, Germany) 3 µL DNA template	1 cycle: 94 ^{0C} ---------- 2 min. 30 cycle: 95 ^{0C} ---------- 30 s 60 ^{0C} ---------- 30 s 72 ^{0C} ---------- 30 s 1 cycle: 72 ^{0C} ---------- 8 min

3. Results

3.1. PCR-Based Detection of *H. pylori*

Figure 1 demonstrates gel electrophoresis results of the PCR method detecting *H. pylori* using 16S rRNA gene sequences.

3.2. SMF-Based Detection of *H. pylori*

Figures 2 and 3 show the SMF findings for the diagnosis of *H. pylori* in the examined biopsy samples.

3.3. Culture-Based Diagnosis of *H. pylori*

Figure 4 shows the treatment group's microbiological culture findings on days 7, 14, 21, and 28 of the experiment. Culture plate showing *H. pylori* colonies in the gastric biopsy samples.

3.4.Comparison of Diagnostic Methods

Table 2 shows the results of three diagnostic methods for *H. pylori* detection in the gastric biopsy samples of experimentally infected mice (treatment group). None of the examined diagnostic tests detected *H. pylori* in the control group. At the beginning of the experiment (day 1), only the SMF technique and PCR could detect the *H. pylori* in the stomach of 3 (60% of mice) and 1 (20% of mice) mice, respectively. On day 7, culture, PCR, and SMF tests detected *H. pylori* in 40%, 80%, and 100% of the examined mice. The SMF detected all infected mice from days 7 to 28 (100%). The PCR test detected all *H. pylori*-infected mice at days 14 to 27 (100%). The other test (culture) detected all infected mice only on day 28 of the experiment. Statistically significant differences were found between the three diagnostic methods on days 0, 7, 14, and 21 of the experiment (P<0.05).

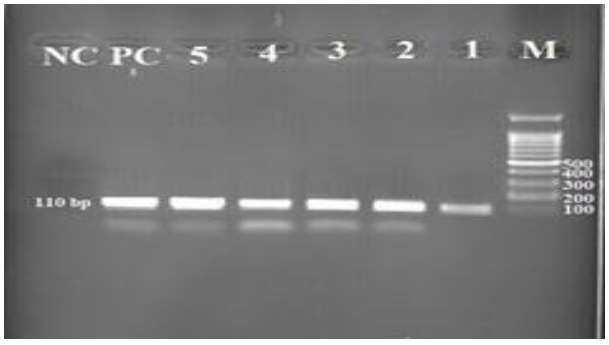

Figure 1. Results of the gel electrophoresis of the PCR method used to detect the *16S rRNA* gene of the *H. pylori*. M: Ladder (100 bp, Thermo Fisher Scientific, St. Leon-Rot, Germany), 1-5: Positive samples for the *16SrRNA* gene of the *H. pylori* (110 bp), PC: Positive control (*H. pylori* ATCC 43504), NC: Negative control (PCR-grade water, Thermo Fisher Scientific, St. Leon-Rot, Germany.)

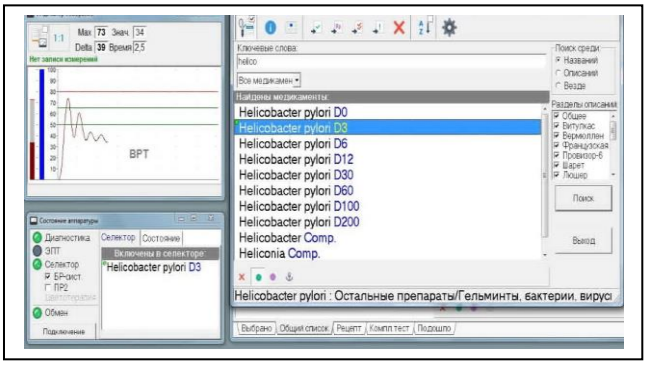

Figure 3. Specificity modulation frequency findings were used to detect *H. pylori* infection in tested biopsy samples. Positive results for *H. pylori* detection

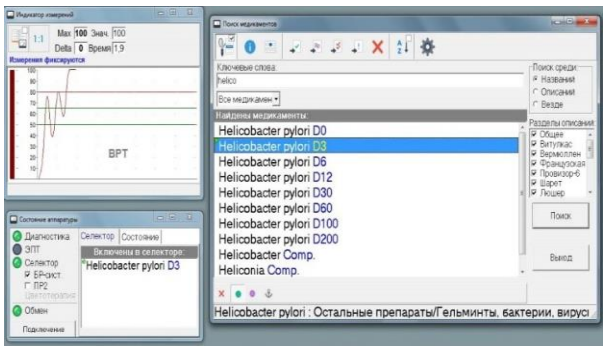

Figure 2. Specificity modulation frequency findings were used to detect *H. pylori* infection in tested biopsy samples. Negative results for *H. pylori* detection

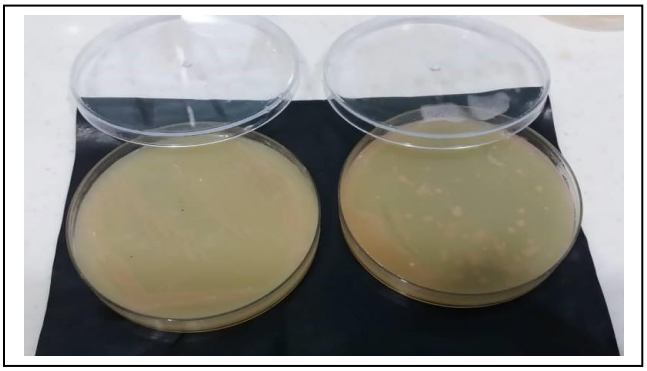

Figure 4. The positive findings of *Helicobacter pylori* infection by microbiological cultures. Culture plate showing *H. pylori* colonies

Table 2. Results of three diagnostic methods for detecting *H. pylori* in the gastric biopsy samples of experimentally infected mice

Days of the experiment	Diagnostic methods (% of positive out of all examined mice)			P-value
	Culture	PCR	Modulation frequency	
0	0/5 [a] (0)	1/5 (20)	3/5 (60)	0.043
7	2/5 (40)	4/5 (80)	5/5 (100)	0.019
14	3/5 (60)	5/5 (100)	5/5 (100)	0.028
21	4/5 (80)	5/5 (100)	5/5 (100)	0.059
28	5/5 (100)	5/5 (100)	5/5 (100)	1.00

A number of positive per total examined mice.

4. Discussion

H. pylori bacteria are one of the most critical infectious pathogens in the human population. As a result, their rapid, safe and sensitive detection is a challenging issue among laboratory practitioners. Several diagnostic approaches have been developed to detect *H. pylori*. Diagnostic tests are either elaborative and need complex and, in some cases, expensive procedures. Some of them are mainly invasive (rapid urease test (RUT), histopathology, culture, and PCR), and others are non-invasive (urease breath test (UBT), fecal antigen test, and some serological and rapid diagnostic tests. However, most of them are limited by low efficiency and accuracy (12).

The purpose of this study was to compare three techniques used to detect *H. pylori*: microbial culture, polymerase chain reaction (PCR) by *16S rRNA* gene sequencing, and SMF technique in the experimentally infected mice model. Our findings revealed the higher ability of the SMF technique for *H. pylori* detection during the experiment. The SMF method detected 60% of the experimentally infected mice on the first day of the experiment. Among other tests, only the PCR detected 20% of the experimentally infected mice at a similar time. On day 7 of the experiment, the SMF method detected all infected mice, while the PCR and culture only can detect 80% and 40% of the infected cases, respectively. The PCR method detected all infected mice on day 14 of the experiment, while the culture detected all the experimentally infected mice only on day 28.

The culture technique used for *H. pylori* diagnosis is time-consuming, and since the bacterium's diagnosis needs colony characters and even further biochemical tests, this technique leftovers are challenging (13). Additionally, *H. pylori* need microaerophilic conditions, a complex culture media with various antibiotics classes, and some supplements with a 5 to 7 days incubation. Besides, the bacterium may turn to a viable form (coccoid) that makes its culture impossible, thus leading to false-negative results (14). Thus, it is not surprising that the present study's applied culture method failed to isolate all bacteria on days 0, 7, 14, and 21 of the experiment.

Biopsy is another diagnostic method for detecting *H. pylori* in microbiological cultures. Since *H. pylori* are highly sensitive to the condition of the external environment, immediate processing of the samples in cultures is necessary. Although the culture method requires more time than other methods, it can be applied to determine *H. pylori* antibiotic resistance. In addition to the time-consuming nature of this method, which can delay results for at least 48 h, its accuracy in detecting *H. pylori* can be substantially influenced by the experience level of laboratory staff (15). Although culture requires endoscopy and dedicated labs, it remains the gold standard for *H. pylori* diagnosis and susceptibility testing. The diagnostic sensitivity and specificity of histology have also been documented; however, a definitive diagnosis of *H. pylori* is difficult by merely observing the morphology of these curved

bacteria under a microscope (16). PCR is only able to identify the sequence of the genome of *H. pylori*. Thus, it cannot separate between live and dead bacteria. Besides, the primer design is the most determining factor in the sensitivity and specificity of PCR (7). It is worth mentioning that DNA-based methods can detect active *H. pylori* and provide a positive response to coccoid forms that may occur in stressful conditions (for example, as a result of consuming antibiotics), the forms that are not detectable by urease-based methods (17).

SMF has recently been developed to diagnose diverse diseases (18). Additionally, SMF has been developed for treating different diseases (19). Considering SMF frequencies, it can be used for various applications ranging from inciting excitable tissues (e.g., the heart, nerves, and muscles) by affecting bone growth and fracture healing to radiofrequency tumor ablation and diathermy (20). The method can detect the vibration and electromagnetic field emitted by the bacteria. In this method, each field spectrum is specific to one application. As a result, there is no possibility of error and cross-reaction.

The last decade brought several *in vivo* and *in vitro* surveys that documented the therapeutic and diagnostic capabilities of electric fields, such as intermittent electric fields, the intermediate frequency with low intensity (100-300 kHz), and electromagnetic fields (EMF) modulated by amplitude at relatively lower frequencies (0.1-114 kHz) (21). According to recent reports (22), SMF application depends on the types of diseases and therapeutic or diagnostic approaches. Otherwise, different frequencies have diverse applications in this technique. As reported in the present study, the SMF technique had the highest efficacy for detecting *H. pylori* in the experiment's early stages.

Khalifehgholi, Shamsipour (23) reported the best sensitivity (95.6%) for the rapid urease test (RUT) and histology. PCR and serology sensitivities were 93.5% and 91.3%, respectively. RUT, PCR, and serology specificities were 100%, 95.6%, and 86.7%, respectively. Ramis, de Moraes (6) reported that the sensitivity of *in-house* urease, PCR, and culture tests was 100%, 100%, and 85.4%, respectively. Khadka, Chapagain (24) reported the accuracy order for *H. pylori* detection as follows: histopathology > RUT > serology > stool antigen test. They concluded that the diagnostic test's accuracy orders might differ depending on the study population and laboratory settings.

Despite the availability of different diagnostic techniques, it leftovers indistinct whether one method could be the gold standard for detecting the infection induced by *H. pylori* (8).

5. Conclusion

To the best of our knowledge, this research is the first attempt to apply SMF technology in detecting *H. pylori* in the mice infected experimentally. SMF may meet the required specification of the gold standard in diagnosing *H. pylori* and may provide the required efficacy in bacterial detection at the early stages of the infection. However, additional research should perform on the other aspects of *H. pylori* detection using the SMF technology.

Authors' Contribution

Study concept and design: S. N. H. A.
Acquisition of data: S. N. H. A.
Analysis and interpretation of data: S. S.
Drafting of the manuscript: M. N. M.
Critical revision of the manuscript for important intellectual content: S. S.
Statistical analysis: S. N. H. A.
Administrative, technical, and material support: S. S.

Ethics

The study design were approved by the ethics committee of Islamic Azad University, Tehran, Iran.

Conflict of Interest

The authors declare that they have no conflict of interest.

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