METHODS AND COMPOSITIONS TO DETECT MICROBES IN FECAL SAMPLES

Abstract: The invention provides a method to test for the presence of a microbe in a fecal sample, a method to determine if the population of a gastrointestinal microbe within an organism increases or decreases over time, a method to test the efficacy of a vaccine against a Helicobacter bacterium, kits, and a nucleic acid segment that specifically hybridizes to H. pylori species found in humans.
For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

Published:
— without international search report and to be republished upon receipt of that report
Methods and compositions to detect microbes in fecal samples

Related Applications

This application claims priority from U.S. Provisional Application Ser. No. 60/468,341 filed May 6, 2003. The entirety of this Provisional Application is incorporated herein by reference.

Statement of Government Rights

This invention was developed with the support of the Department of Health and Human Services. The United States Government has certain rights in the invention.

Field of the Invention

The invention generally relates to methods to isolate nucleic acid from fecal samples that may be specifically amplified. The invention also relates to methods, nucleic acid primers, and kits that may be used to detect Helicobacter bacteria, such as H. pylori.

Background of the Invention

Helicobacter pylori is a bacterium that has been established as a major gastroduodenal pathogen. It is estimated that more than 50% of the world population is infected with this pathogen which causes gastritis of varying severity. H. pylori infection causes overt disease in a large number of infected persons. For example, the majority (95%) of duodenal ulcers are associated with H. pylori infection. A causal role of H. pylori in producing duodenal ulcers has been shown by studies which indicate that ulcers do not recur if H. pylori is eradicated at the time of ulcer healing. In contrast, there is an 80% recurrence rate at one year in those patients who remain infected with H. pylori. Furthermore, up to 80% of gastric ulcers are thought to be H. pylori associated (Blaser, Clin. Infect. Dis., 15:386 (1992)).

There is now increasing evidence of the harmful consequence of long term H. pylori infection. Persons who have become infected with the bacterium
early in life show a slow progression of gastritis over approximately 30-40 years of continual infection before severe gastric atrophy appears.

Gastric atrophy is well documented as being the precursor lesion leading to gastric cancer. The actual cancer that develops in an atrophied stomach is dependent on a myriad of other factors that include diet. However, evidence to date suggests that often times the cancer would not have developed if it was possible to remove the H. pylori infection at an early age before the atrophy had developed (Parsonnet et al., J. Natl. Cancer Inst., 19:881 (1991)).

Methods that can be used to detect an H. pylori infection are very valuable to allow appropriate diagnosis and treatment of persons who are infected with H. pylori. A number of methods that may be used to detect H. pylori are currently available.

For example, endoscopy of the stomach can be performed in order to obtain a tissue biopsy. The biopsy can then be examined by a number of methods, including microscopy and histological methods. Unfortunately, this procedure has a number of drawbacks. First, it is highly invasive. Second, the patient must be sedated during the procedure. Third, the test cannot measure Helicobacter pylori activity in "real time". That is, there is a significant delay between the time the Helicobacter pylori activity takes place, and the time such activity is measured by the test.

A blood test may also be used to detect an H. pylori infection in a patient. A sample of blood is withdrawn and tested for the presence of antibodies to Helicobacter pylori. However, because antibodies can remain in the body for 6-24 months following exposure to H. pylori, this test does not indicate whether a patient is currently infected or if the infection has been eradicated following treatment.

Another detection method is a breath test. This method is generally based on the activity of Helicobacter pylori in producing a large quantity of the enzyme urease, which hydrolyzes urea to form carbon dioxide and ammonia. Thus, this method involves orally administering isotopically-labeled or radioactively-labeled urea to a subject, and then analyzing the exhaled breath of the subject for the presence of isotopically-labeled or radio-labeled carbon dioxide or ammonia. The presence of these labeled compounds indicates the
presence of an infection in the gastrointestinal tract of the subject. Unfortunately, this method requires ingestion of an isotopically-labeled or a radioactively-labeled compound.

Another drawback of detection methods for H. pylori that are based on urease activity is that numerous species of Helicobacter exhibit urease activity. Thus, urease based methods are not able to distinguish one Helicobacter species from another.

Accordingly, methods are needed that allow rapid general diagnosis of Helicobacter infection, and methods that can be used to specifically detect H. pylori infection.

Summary of the Invention

The invention provides a method to test for the presence of a microbe in a fecal sample, a method to determine if the population of a gastrointestinal microbe within an organism increases or decreases over time, a method to test the efficacy of a vaccine against a Helicobacter bacterium, kits, and a nucleic acid segment that specifically hybridizes to H. pylori species found in humans.

The invention provides a method to test for the presence of a microbe in a fecal sample obtained from an organism. Generally, the method involves isolating amplifiable nucleic acid from a fecal sample obtained from an organism, and determining if the nucleic acid is specific for the microbe. Preferably the microbe is a bacterium. More preferably the microbe is a gram-positive bacterium. Even more preferably the microbe is a gram-negative bacterium. Still even more preferably the microbe is a member of the genus Helicobacter. Most preferably the microbe is Helicobacter pylori. Preferably the organism is a mammal. More preferably the organism is a commercial mammal. Most preferably the organism is a human. A nucleic acid amplification process can be used to determine if the nucleic acid is specific for a microbe. Preferably the nucleic acid amplification process is the polymerase chain reaction. More preferably the nucleic acid amplification process is the polymerase chain reaction with a Helicobacter genus specific primer. Most preferably the nucleic acid amplification process is the polymerase chain reaction with a Helicobacter pylori specific primer.
The invention provides a method to determine if the population of a gastrointestinal microbe within an organism increases or decreases over time. Generally, the method involves determining an amount of nucleic acid that is specific for a microbe in a first fecal sample obtained from the organism at a first time point and in a second fecal sample obtained from the organism at a second time point, and comparing the amount of nucleic acid present in the first fecal sample to the amount of nucleic acid present in the second fecal sample. Preferably the microbe is a bacterium. More preferably the microbe is a gram-positive bacterium. Even more preferably the microbe is a gram-negative bacterium. Still even more preferably the microbe is a member of the genus Helicobacter. Most preferably the microbe is Helicobacter pylori. Preferably the organism is a mammal. More preferably the organism is a commercial mammal. Most preferably the organism is a human. A nucleic acid amplification process can be used to determine if the nucleic acid is specific for a microbe. Preferably the nucleic acid amplification process is the polymerase chain reaction. More preferably the nucleic acid amplification process is the polymerase chain reaction with a Helicobacter genus specific primer. Most preferably the nucleic acid amplification process is the polymerase chain reaction with a Helicobacter pylori specific primer. Preferably the method is used to determine whether administration of a therapeutic agent increases or decreases the population of a gastrointestinal microbe.

The invention provides a method to test the efficacy of a vaccine against a Helicobacter bacterium. Generally, the method involves obtaining a fecal sample from a first vaccinated organism that was inoculated with Helicobacter bacteria, and determining if the fecal sample contains an increased or decreased amount of Helicobacter specific nucleic acid when compared to an amount of Helicobacter specific nucleic acid contained within a fecal sample obtained from a non-vaccinated organism that was inoculated with the Helicobacter bacteria. Preferably the Helicobacter bacteria is Helicobacter pylori. A nucleic acid amplification process can be used to determine if a fecal sample contains Helicobacter specific nucleic acid. Preferably the nucleic acid amplification process is ligation activated transcription. More preferably the nucleic acid amplification process is ligase chain reaction. Most preferably the nucleic acid
amplification process is polymerase chain reaction. A Helicobacter specific primer can be used during the nucleic acid amplification process. Preferably the Helicobacter specific primer is a Helicobacter genus specific primer. More preferably the Helicobacter specific primer is a Helicobacter pylori specific primer. Preferably the organism is a mammal. More preferably the organism is a commercial mammal. Most preferably the organism is a human.

The invention provides kits. A kit of the invention includes packaging material, including instructions for use, and a Helicobacter specific primer. Preferably the Helicobacter specific primer is a Helicobacter genus specific primer. More preferably the Helicobacter specific primer is a Helicobacter pylori specific primer. Even more preferably the Helicobacter specific primer includes a nucleotide sequence corresponding to SEQ ID NO:1. Most preferably the Helicobacter specific primer has a nucleotide sequence that corresponds to SEQ ID NO:1. A Helicobacter specific primer may be linked to a detectable label. Preferably the detectable label is a radioactive label. More preferably the detectable label is a non-radioactive label. A kit of the invention can optionally include a support that can be used for isolation of nucleic acid. Preferably the support is a silica-based support. A kit of the invention can optionally include buffer that can be used for isolation of nucleic acid. A kit of the invention can optionally include a thermostable polymerase. Preferably the thermostable polymerase can be used for polymerase chain reaction amplification of a nucleic acid template.

The invention provides nucleic acid segments that specifically hybridize to H. pylori species found in humans. Preferably these nucleic acid segments include SEQ ID NO:1. More preferably a nucleic acid segment has a nucleotide sequence that corresponds to SEQ ID NO:1.

Brief Description of the Drawings

Figures 1A-1B. Figure 1A, side A illustrates the specificity of the species specific primer (Hp1-Hp2) by showing that only H. pylori SS1 DNA is PCR- amplified to produce a 109 bp fragment (See arrows). Lane 1, H. pylori DNA; Lane 2: H. bilis DNA; Lane 3, H. hepaticus DNA; Lane 4, H. muridarum DNA; Lane M, 100 bp DNA Marker. Figure 1A, side B shows PCR detection
of Helicobacter species with genus specific primers (16S1 16S2) and electrophoretic analysis of 375 bp PCR products. Lane 6, PCR reagents control; Lane 7, H. pylori SS1; Lane 8, H. bilis DNA; Lane 9, H. hepaticus DNA; Lane 10, H. muridarum DNA. Figure 1B, sides C-D show the specificity and selectivity of the species-specific primers (Hp1-Hp2) in the presence of genomic DNA of three other Helicobacter species and fecal material. Only H. pylori SS1 DNA was PCR-amplified, producing a 109 bp fragment in the presence of other Helicobacter species genomic DNA’s and fecal material (See arrow lanes 1-4). Lane 1, 100 bp ladder; Lane 2, A 10X dilution of a DNA mixture containing 9 ng/μl of genomic DNA from four Helicobacter species (H. pylori SS1, H. bilis, H. hepaticus, and H. muridarum); Lane 3, 100X dilution of the same DNA mixture; Lane 4, 1000X dilution of the same DNA mixture; Lane 5, A 10X dilution of a DNA mixture (9 ng/μl of genomic DNA per organism) containing only H. bilis, H. hepaticus, and H. muridarum; Lane 6, PCR reagent control. D. PCR amplification of four Helicobacter species genomic DNA’s with genus specific primers (16S1 16S2) in the presence of fecal material. Lane 8, 10X dilution of DNA mixture described above; Lane 9, 100X dilution; Lane 10, 1000X dilution; Lane 11, 10X dilution of a DNA mixture minus H. pylori SS1 genomic DNA and Lane 12, PCR reagent control.

Figures 2A-B. Figure 2A illustrates the sensitivity of the PCR assay. (Figure 2A) DNA extracted from serial dilutions (10^5 – 10^0 CFU/ml) of H. pylori SS1 culture and amplified with species-specific primer set Hp1-Hp2; (Figure 2B) DNA extracted from serial dilutions (10^5 – 10^0 CFU/ml) of H. pylori SS1 culture in the presence of fecal pellet and also amplified with the primer set Hp1-Hp2. The arrows indicate the 109 base pair PCR products.

Figure 3 shows PCR products of DNA extracted from mouse fecal pellets resolved by 2% agarose gel electrophoresis. PCR was performed with species-specific primers (Hp1-Hp2), giving 109 bp products as indicated by the arrow. Lane 1, positive control (H. pylori SS1 DNA); Lane 2, negative control; Lanes 3, 5, 6, 7 and 8, Fecal pellets from H. pylori SS1 infected mice; Lane 4, Fecal pellet from an uninfected mouse.

Figure 4 illustrates Helicobacter pylori SS1 genomic fingerprinting using the RAPD method. Test performed with 10 nt primers 1281 (Lanes 1 & 2)
and 1290 (Lanes 4 & 5) confirmed RAPD profile identity of bacterial strain recovered from the mice to profile of strain used for inoculation. Lanes 1 & 4, H. pylori SS1 recovered from infected mice; Lane M, 1 Kb ladder; Lanes 2 & 5, H. pylori SS1 positive control.

Figures 5A-B. Figure 5 shows mouse gastric tissue (Corpus; Genta and H&E stains; original magnification 100X for main picture, 1000X for inset). (Figure 5A) Sham-inoculated mouse (Genta). (Figure 5B) Mouse 6 months after inoculation with H. pylori strain SS1 (H&E). Note minimal infiltration (arrow). The inset shows H. pylori near epithelial surface. (Figure 5C) Mouse 10 months after inoculation with strain SS1 (Genta). Note marked infiltration by inflammatory cells (arrow). The inset shows numerous bacteria near epithelial surface (original magnification 1000X).

Detailed Description of the Invention

Methods to detect and identify microbes play an important role in everyday life. For example, detection and identification of microbes is used for medical diagnosis and treatment of infections, determining the effectiveness of a medical treatment scheme, or determining whether prophylactic measures taken to prevent infection with a microbe are effective. Improved methods to detect microbes that decrease the time necessary to obtain results, or increase the accuracy of the results obtained, can save the lives of persons infected with such microbes. Improved detection methods also allow clinicians to respond to infections more rapidly with appropriate treatment schemes.

The present invention provides methods to detect infection of an organism by a microbe through collection of a fecal sample from the organism, and determining if the fecal sample contains nucleic acid that is specific for a microbe. For example, the methods of the invention can be used to determine if an organism is infected with Helicobacter bacteria, such as Helicobacter pylori.

Experiments conducted according to the invention and disclosed herein were validated in vitro by demonstrating that they are specific and sensitive for the detection of a microbe, Helicobacter pylori for example, in aqueous solutions, and also in fecal pellets. Experiments conducted according to the invention were also validated in vivo by demonstrating that PCR of the fecal
pellets was positive in animals that tested positive by histology, culture, and/or PCR of gastric mucosal samples that were obtained when the animals were sacrificed. Moreover, PCR of the fecal pellets was negative in all sham-inoculated animals used as controls.

Four of the test animals that were inoculated with Helicobacter pylori tested positive by PCR of the fecal pellets, histology, and PCR of gastric specimens, but were negative by culture. These results suggested that culture based methods are the least sensitive method for assaying for Helicobacter infection when compared to the three other tests. One of the animals was positive by culture and stool PCR, but negative when assayed through use of histological based methods. These results indicate that the PCR based methods of the invention are at least as sensitive and reliable as detection methods based on histological examination, culturing samples, and PCR of gastric samples.

A beneficial aspect of a method of the invention is that samples may be tested for the presence of a microbe in a manner that is non-invasive to the organism being tested. This allows multiple samples to be obtained and tested at different times. Such a sampling procedure allows the progression of a microbial infection to be followed over time with a minimum of discomfort to the organism being tested. This is particularly beneficial in the case of laboratory animals, such as mice, that have been previously sacrificed when tested for Helicobacter infection. For example, previous PCR based assays for the detection of H. pylori in mice have been described that required euthanasia of the experimental animals. Use of a method of the invention allows H. pylori detection in a mouse without having to euthanize the mouse.

Another beneficial aspect of a method of the invention is that inhibitors of nucleic acid amplification methods are eliminated through use of the methods of the invention. To overcome difficulties due to the presence of inhibitors in fecal samples, two steps were introduced in the fecal DNA preparation: (I) use of a silica-based column which binds the nucleic acid, thus allowing for extensive wash of the columns in order to remove most, if not all, inhibitors; and (II) dilution of samples (1:10, 1:20, and 1:40) to be used as template. This approach reduced the effect of inhibitory substances present in the DNA
extracted from fecal matter and allowed use of the polymerase chain reaction to amplify microbe specific nucleic acid.

Definitions:

Abbreviations: Bisulfiteless Brucella Broth (BLBB), Glaxo Selective Supplement-A (GSSA), Sydney Strain 1 (SS1), Mucosal Associated Lymphoid Tissues (MALT), Polymerase Chain Reaction (PCR), Random Amplified Polymorphic DNA (RAPD), fluorescence resonance energy transfer (FRET), ribonucleic acid (RNA), deoxyribonucleic acid (DNA).

The term "amplifluor" refers to a primer with a hairpin structure at the 5' end that contains a fluorophore and a quencher. Incorporation of the amplifluor into an amplified nucleic acid opens the hairpin to produce fluorescence (Nazerenko et al., Nucleic Acids Res., 25:2516 (1997)). An amplifluor as used herein can be synthesized such that it will hybridize to nearly any nucleic acid sequence desired. Accordingly, amplifluors can be synthesized that specifically recognize nucleic acid obtained from members of the Helicobacter genus, or nucleic acid obtained from Helicobacter pylori.

A "commercial mammal" is a mammal that is produced for the generation of revenue. Examples of commercial mammals include, pigs, sheep, dogs, cats, cattle, horses, goats, mink, buffalo, and the like.

A "Helicobacter pylori" specific primer refers to an oligonucleotide that specifically hybridizes to nucleic acid obtained from Helicobacter pylori as opposed to other members of the Helicobacter genus. Accordingly, a Helicobacter pylori specific primer can be used to identify a bacterium that is member of the Helicobacter genus as Helicobacter pylori. An example of a Helicobacter pylori specific primer has the nucleotide sequence 5'-AACATTACTGACGCTGATTG-3' (SEQ ID NO:1). A Helicobacter pylori specific primer may also include nucleotide sequences that are not complimentary to deoxyribonucleic acid obtained from Helicobacter pylori. For example, a Helicobacter pylori specific primer can be an amplifluor, a scorpion primer, or a molecular beacon.

A "molecular beacon" is a nucleic acid sequence that contains a target recognition loop flanked by a hairpin with a fluorophore and quencher on the
The opposite ends of the nucleic acid sequence. Specific binding of the molecular beacon to a target nucleic acid opens the hairpin and separates the fluorophore from the quencher to produce fluorescence (Tyagi and Kramer, Nat. Biotechnol., 14:303 (1996)). Molecular beacons are known and have been described (Schweitzer and Kingsmore, Curr. Opin. Biotech., 12:21-27 (2001); Piatek et al., Antimicrob. Agents Chemother., 44:103 (2000); McKillip and Drake, J. Food Prot., 63:855 (2000)). A molecular beacon can be constructed to include nearly any nucleic acid sequence that specifically binds to a target site. For example, a target site may be a nucleic acid sequence that specifically hybridizes to nucleic acid from members of the Helicobacter genus, or that more specifically hybridizes to nucleic acid from Helicobacter pylori.

The term "primer" as used herein refers to an oligonucleotide that can hybridize to a complementary nucleic acid sequence. Primers are usually between 7 and 15 nucleotides in length. However, primers may be created that are longer or shorter depending on their intended use.

A "scorpion primer" as used herein refers to a nucleic acid primer that includes a first portion having a nucleotide sequence that specifically hybridizes to a target nucleic acid sequence, a second portion that forms a hairpin, a third portion having a nucleotide sequence that specifically hybridizes to a target nucleic acid sequence, a quenching agent, and a fluorescent agent. The first portion of the scorpion primer will specifically anneal to a target sequence to provide a 3' end that can be extended by a polymerase. Extension of the 3' end of the scorpion primer produces an oligonucleotide having a nucleotide sequence to which the third portion hybridizes. Hybridization of the third portion disrupts the hairpin of the second portion and causes separation of the fluorescent agent from the quenching agent to produce a fluorescent signal. Scorpion primers have been described (Thelwell et al., Nucleic Acids Res., 28:3752 (2000); Schweitzer and Kingsmore, Curr. Opinion Biotech., 12:21 (2001)). Scorpion primers can be prepared that contain nearly any nucleotide sequence that specifically hybridizes to a target nucleic acid sequence. For example, scorpion primers can be prepared that contain Helicobacter genus specific nucleic acid sequences, or Helicobacter pylori specific nucleic acid sequences.
The term "therapeutic agent" includes chemical compounds and biologicals that can be used to treat an infection of an organism by a microbe. Examples of chemical compounds include aminoglycosides, amphenicols, ansamycins, beta-lactams, lincosamides, macrolides, polypeptides, tetracyclines, 2,4-diaminopyrimidins, nitrofurans, oxazolidines, quinolones, sulfonamides, sulfones, and the like. Numerous therapeutic agents are known in the art and have been reported (Merck Index, 13th Edition, Merck & Co., Whitehouse Station, NJ (2001)).

The term "selectively hybridize" as used herein refers to the ability of an oligonucleotide primer to hybridize to a preselected nucleic acid and not hybridize to other nucleic acid sequences. For example, a primer that selectively hybridizes to nucleic acid isolated from a member of the Helicobacter genus will not hybridize to nucleic acid isolated from members of the Escherichia genus. In another example, a primer that selectively hybridizes to nucleic acid isolated from Helicobacter pylori will not hybridize to nucleic acid isolated from Helicobacter canis.

A "taqman primer" is an oligonucleotide primer that is 3'-labeled with a fluorophore and 5'-labeled with a fluorescent quencher that, upon binding to an amplification product, undergoes quencher removal by the 5'-3' exonuclease activity of Taq DNA polymerase. Removal of the quencher produces a fluorescent signal to indicate the presence of nucleic acid to which the taqman primer can hybridize.

A "thermostable ligase" as used herein refers to an enzyme that catalyzes the formation of a phosphodiester bond between juxtaposed 5' phosphate and 3' hydroxyl termini if two adjacent oligonucleotides which are hybridized to a complementary target deoxyribonucleic acid, and is stable at elevated temperatures. Such ligases are commercially available (New England Biolabs, Beverly, MA).

A "thermostable polymerase" as used herein refers to an enzyme that catalyzes the 5' to 3' addition of a nucleotide onto the end of an oligonucleotide, and that is stable at elevated temperatures. Such polymerases are commercially available (New England Biolabs, Beverly, MA).
A. A method to test for the presence of a microbe in a fecal sample

The invention provides a method to test for the presence of a microbe in a fecal sample. The method generally involves isolating amplifiable nucleic acid from the fecal sample, and determining if the amplifiable nucleic acid includes a nucleic acid segment having a nucleotide sequence that is specific for the microbe.

Amplifiable nucleic acid can be isolated from a fecal sample through use of a support to which nucleic acid present in a fecal sample is initially bound. A fecal sample can be contacted directly with the support so that nucleic acid within the fecal sample can bind to the support. Alternatively, the fecal sample can be treated to disrupt microbes within the fecal sample so that nucleic acid contained within the microbes is released and is able to be bound to the support. The nucleic acid that is bound to the support is then washed to separate fecal components from the bound nucleic acid. Elimination of fecal components from the bound nucleic acid that are inhibitory to nucleic acid amplification processes produces amplifiable nucleic acid.

Numerous supports may be used to produce amplifiable nucleic acid. For example, an anion-exchange support may be used to produce amplifiable nucleic acid. In another example, the support can be a silica-based support. In another example, hydroxyapatite can be used as a support. Such supports are known in the art and are commercially available (Qiagen, Valencia, CA; Pharmacia, Piscataway, NJ). The supports can be used according to numerous methods to bind nucleic acid and eliminate fecal components from the bound nucleic acid. In one example, the support may be prepared as a column to facilitate washing of the support. In another example, the support can be used in a slurry to wash the bound nucleic acid.

The bound nucleic acid can be washed with numerous types of fluids. These fluids may contain components that act as pH buffers. The fluids may also contain components that facilitate separation of fecal components from nucleic acids that are bound to a support. For example, a fluid used to wash nucleic acid bound to a support may contain detergents, salts, enzymes (such as lipases), or other components that degrade or separate fecal components from bound nucleic acids.
Nucleic acid separated from a fecal sample can be classified as amplifiable nucleic acid by determining if the treated DNA can serve as a template in an amplification reaction. Examples of such amplification reactions include, polymerase chain reaction, ligase chain reaction, and the like.

A nucleic acid segment that is specific for a microbe may be determined through detection of an amplification product that is produced by use of the nucleic acid segment as a template. The amplification product may be detected through use of a radioactive label. In one example, an α-labeled nucleotide can be included within an amplification reaction to produce an internally labeled amplification product. In another example, an end-labeled primer may be included within an amplification reaction to produce an end-labeled amplification product. Numerous methods for producing radioactively labeled amplification products are known in the art and have been described (Sambrook et al., Molecular Cloning: A Laboratory Manual, 3rd edition, Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (2001)). An amplification product may be detected through use of a non-radioactive label. Numerous non-radioactive labels are known and have been described (Sambrook et al., 2001). Examples of such non-radioactive labels include dinitrophenol, bromodeoxyuridine, biotin, digoxigenin, fluorescein, and the like (Guesdon, J. Immunol. Methods, 150:33 (1992); Viale and Dell'Orto et al., Liver, 12:243 (1992); Reischl et al., Mol. Biotechnol., 1:229 (1994); Mansfield et al., Mol. Cell. Probes, 9:145 (1995)). An amplification product may also be detected through hybridization of a probe to the amplification product as is known in the art. In one example the amplification product is detected by hybridizing an amplifluor to the amplification product. In another example, the amplification product is detected by hybridizing a molecular beacon that contains a Helicobacter genus specific nucleotide sequence, or a Helicobacter pylori specific nucleotide sequence to the amplification product. The amplification product may also be detected through use of a Taqman primer. Fluorescence resonance energy transfer (FRET) may be used to detect a nucleic acid segment having a nucleotide sequence that is specific for the microbe, or an amplification product. Briefly, Fluorescence energy transfer is a distance-dependent interaction between the electronic excited states of two dye molecules in which excitation is transferred
from a donor molecule to an acceptor molecule. The efficiency of FRET is
dependent on the inverse sixth power of the intermolecular separation, making it
useful over distances comparable with the dimensions of biological
macromolecules. Typically, a donor and an acceptor molecule are within from
10-100 angstroms of each other. Typical examples of donor and acceptor
molecule pairs include: fluorescein-tetramethylrhodamine, IAEDANS-
fluorescein, EDANS-Dabcyl, fluorescein-fluorescein, BODIPY FL-BODIPY
FL, fluorescein-QSY 7 dye, and fluoreescein-QSY 9 dye. Dyes and instructions
for their use are commercially available and known in the art (Molecular
Probes, Inc., Eugene OR, 97402). Accordingly, a first nucleotide sequence
specific primer can be labeled with a first dye, and a second nucleotide
sequence specific primer can be labeled with a second dye so that the first and
second primers will anneal to a microbe specific nucleotide sequence. The
annealed primers can then be ligated through the action of ligase to produce a
fluorescent signal indicative of the specific microbial nucleotide sequence. The
microbe specific nucleotide sequence can be included in the amplifiable nucleic
acid separated from a fecal sample, or in an amplification product.

These methods may be used with automated systems for high-
throughput assays to screen samples, such as clinical samples, for the presence
of a microbe or microbes. Automated methods are known in the pharmaceutical
industry. For example, fluorimetric imaging plate readers (FLIPR) may be used
in conjunction with robotic arms to determine if a fecal sample contains a
specific microbe or microbes. The robotic arms can be used to add components
to a 96 well plate to separate amplifiable nucleic acid from fecal samples,
conduct amplification reactions, and determine if the amplifiable nucleic acid
contains a nucleic acid segment that is specific for a microbe. Scorpion primers
or molecular beacons may be particularly suitable for use with automated
systems due to their ease of use, specificity, ease of detection, and stability.

B. A method to determine if a population of a gastrointestinal microbe
within an organism increased or decreased during a time period

Generally the method involves determining an amount of nucleic acid
that is specific for a microbe in a first fecal sample obtained from the organism
at a first time point and in a second fecal sample obtained from the organism at a second time point, and comparing the amount of nucleic acid present in the first fecal sample to the amount of nucleic acid present in the second fecal sample.

Nucleic acid that is specific for a microbe can be determined as described above. The amount of nucleic acid present in the first sample and in the second sample can each be determined through use of quantitative methods, such as analytical PCR. Alternatively, the relative amount of nucleic acid that is specific for a microbe in the first sample and in the second sample can be compared. Comparison of a relative amount of specific nucleic acid involves comparing an amount of specific nucleic acid in a first sample and in a second sample that is normalized to a constant standard. For example, the amount of specific nucleic acid in the first and second sample may be normalized to the total mass of fecal material from which the specific nucleic acid was separated.

Alternatively, the specific nucleic acid may be normalized to the total amount of nucleic acid separated from a fecal sample. In another alternative, PCR amplification reactions can be prepared that include a control nucleic acid template and a set of primers that anneal to the control nucleic acid template. Thus, the amount of the control template that is amplified during the PCR reactions can serve as an internal control that can be used to compare the amount of a specific nucleic acid in a first sample to the amount of a specific nucleic acid in a second sample. Those of skill can readily select an appropriate standard with which to compare relative amounts of specific nucleic acid from a first and second sample.

The method of the invention can be used to monitor an increase or decrease in microbial population over time. Accordingly, the method is useful for assessing the effectiveness of treatment schemes designed to reduce or eliminate the presence of microbes within an organism. For example, the method may be used to assess the effectiveness of antibiotics administered to a patient to treat a Helicobacter pylori infection. In another example, the method can be used to determine if the prophylactic administration of antibiotics is effective to prevent infection of an organism with a microbe. Such applications
may be suitable for use in the agricultural industry in the field of animal husbandry.

The method can also be used to screen therapeutic agents for their ability to decrease Helicobacter infection of an organism. For example, a series for mice could be inoculated with Helicobacter bacteria, and then treated with a candidate agent. Fecal samples could be collected from the individual mice and the effect of the candidate agent on the population of Helicobacter bacteria within the mice could be determined. Such a method would allow therapeutic agents to be identified that could be used to treat Helicobacter infections, such as infections by Helicobacter pylori.

C. A method to test efficacy of a vaccine against infection by Helicobacter bacteria

Generally the method involves obtaining a fecal sample from a first vaccinated organism that was inoculated with Helicobacter bacteria, and determining if the fecal sample contains an increased or decreased amount of Helicobacter specific nucleic acid when compared to an amount of Helicobacter specific nucleic acid contained within a fecal sample obtained from a non-vaccinated organism that was inoculated with the Helicobacter bacteria.

Methods to vaccinate organisms against infection by Helicobacter bacteria have been described (Prinz et al., Trends Microbiol., 11:134 (2003); Eisenberg et al., Infect. Immun., 71:1820 (2003)). Briefly, an organism may be vaccinated to decrease Helicobacter infection by administering killed Helicobacter to the organism in the presence of an adjuvant. In addition, Helicobacter proteins, or protein subunits, may be administered to the organism in the presence of an adjuvant. General methods for vaccinating organisms are known in the art and have been reported.

Use of the method of the invention allows the efficacy of a vaccine to be tested in a non-invasive manner. Thus, the ability of a vaccinated organism to decrease or resist infection by a member of the Helicobacter genus can be tested at multiple time points with a minimum of discomfort. This provides a rapid screening test that can be used to test multiple vaccines for their ability to
produce an effective immune response against Helicobacter bacteria in an organism.

D. **A kit comprising packaging material and a Helicobacter pylori specific primer.**

The invention provides a kit that includes packaging material, including instructions for use, and a Helicobacter specific primer. Primers that can be included within a kit of the invention include those having SEQ ID NOs: 1-7. In addition, primers that selectively hybridize to nucleic acid isolated from members of the Helicobacter genus, and that selectively hybridize to nucleic acid isolated from Helicobacter pylori can be included within the kits of the invention. The primers may be radioactively labeled or non-radioactively labeled. The primers can include, but are not limited to, scorpion primers, molecular beacons, Taqman primers, and amplifluors. A kit of the invention can also include a support to which nucleic acid binds to allow separation of the nucleic acid from fecal components. A kit of the invention may also include a fluid used to separate fecal components from nucleic acid that is bound to a support. A kit of the invention can also include a thermostable polymerase or a thermostable ligase, and buffers that can be used with a polymerase or ligase. A kit of the invention can also include nucleotides that can be incorporated into an amplification product.

**Examples**

**Bacterial strains and growth conditions**

The H. pylori strain used herein was Sydney Strain 1 (SS1) (Lee et al., *Gastroenterology*, 112:1386 (1997)). The strain was grown for 16 to 18 hours at 37 °C in a microaerophilic atmosphere and in bisulfiteless Brucella broth (BLBB) (Hawrlik et al., *J. Clin. Microbiol.*, 32:790 (1994)) containing 10% fetal bovine serum (HyClone, Logan, UT). For solid medium, 1.5% agar was added to BLBB broth. Cultures were tested for urease, catalase, and oxidase activities. The cultures were also monitored microscopically, for example by use of Gram staining (Montgomery et al., *Am. J. Clin. Path.*, 90:606 (1998); Pinkard et al., *J. Clin. Pathol.*, 39:112 (1986)). Helicobacter bilis (ATCC #
51632), H. hepaticus (ATCC # 51449), and H. muridarum (ATCC # 51212) stocks were obtained from the American Type Culture Collection (Manassas, VA).

**Isolation of DNA from bacterial strains**

Genomic DNA was extracted from H. pylori, H. bilis, H. muridarum, and H. hepaticus through use of a commercially available kit (Wizard Genomic DNA Purification Kit; Promega, Madison, WI). The ATCC stocks of H. bilis, H. muridarum, and H. hepaticus were hydrated in 1.5 ml of BLBB medium and subjected to the extraction protocol as described by the manufacturer.

**Animal Housing and Diet**

The mice were maintained in a National Institutes of Health (NIH) animal facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International (Rockville, Maryland). The mice were maintained in a specific pathogen-free animal care holding room and were confirmed to be free of the following microorganisms: CAR Bacillus, Ectromelia, Mouse Rotavirus, Mouse Encephalomyelitis Virus, Lymphocytic Choriomeningitis Virus, Murine Cytomegalovirus, Mouse Hepatitis Virus, Mouse Adenovirus, Minute Virus of Mice, Mycoplasma pulmonis, Parvovirus, Polyoma Virus, Pneumonia Virus of Mice, Reovirus 3, and Sendai Virus.

Mice were housed in 7.5 x 11.5 x 5-in. sterilized ventilated Thoren cages (Thoren Caging System, Inc, Hazleton, PA) on Tek Fresh bedding (Harlan Teklad, Madison, WI). Cages were changed weekly. The animal holding room was maintained under environmental conditions of 20°C, 40 to 70% relative humidity, 15 air changes/h and a 12:12-h light:dark cycle. Mice were fed autoclaved pelleted rodent diet (Rodent NIH-31 Autoclavable NA, Zeigler Brothers, Inc., Gardners, Pennsylvania) ad libitum and provided sterilized individual water bottles for ad libitum water source. On arrival, the mice were acclimated a minimum of 7 days prior to being used in the experiments. Mice were identified using numerical stainless steel rodent ear tags (National Band and Tag, Co., Newport, Kentucky). This study was reviewed and approved by the NIH Institutional Animal Care and Use Committee. All procedures and use
of animals were in compliance with the Public Health Service Guide for the Care and Use of Laboratory Animals.

Animal inoculations and follow-up

Forty-two 6- to 8-week old Helicobacter-free and pathogen-free female C57BL/6 mice (N=42) (Charles Rivers, Wilmington, Massachusetts, USA) were used in compliance with guidelines and protocols approved by the Animal Care and Use Committee of the National Institutes of Health, USA. A 20-gauge ball-point metal feeding tube (Harvard Apparatus, Inc., Holliston, MA) was used to inoculate 26 mice intragastrically with 0.1 ml of a H. pylori Sydney Strain-1 cell suspension (108 colony-forming-units per milliliter – CFU/ml). The cell suspension was created from three separate overnight cultures on three alternate days. Sixteen control mice were inoculated with BLBB containing 10% fetal bovine serum. All animals were housed in a pathogen-free environment in ventilated cages with sterilized beddings, feedings, and water. The mice were repeatedly tested for the presence of H. pylori by PCR analysis of DNA extracted from the fecal pellets. During necropsy at 6 and 10 months, stools were again tested by PCR and stomachs were harvested for H. pylori recovery, histology, and PCR.

Isolation of DNA from fecal pellets

Fecal pellets were collected by holding a mouse above a sterile microfuge tube and gently stroking the lower left side of the abdomen. The tubes were placed on ice and processed immediately. DNA was isolated from fecal pellets (2 pellets per mouse) through use of commercially available instructions and materials (Qiagen DNeasy Kit or DNA Stool Kit; Valencia, CA). The pellets were first suspended in the proteinase-K reaction buffer (250 µl) provided in the kit and homogenized with a sterile motorized pestle (Daigger, Lincolnshire, IL). The subsequent steps were as outlined by the manufacturer for purification of DNA from animal tissue or stool, with the exception that the volume of the column wash solution was increased 3-fold prior to DNA elution.
Polymerase Chain Reaction (PCR)

PCR templates: DNA samples (unquantitated) that were extracted from the fecal pellets were diluted 1:10, 1:20, and 1:40. Five to 15 μl of the dilutions were used as template in the PCR reaction with H. pylori-specific primers (see below). The dilutions allowed input of an optimum amount of template DNA, and reduced the effect of PCR-inhibitory substances (Monteiro et al., J. Clin. Microbiol., 35:995 (1997)).

PCR primers: Genus-specific primers, 16S1 [5'-CTATGACGGGTATCCGGC] (SEQ ID NO:2) and 16S2 [5'-ATTCCACCTACCTCTCCCA] (SEQ ID NO:3) (Riley et al., J. Clin. Microbiol., 34:942 (1996)), directed at conserved regions of the 16S rRNA gene of the Helicobacter genus were used in this study. These primers amplify a 375-bp product. H. pylori species-specific primers, Hp1 [5'-CTGGAGAGACATAGCCCTCC] (SEQ ID NO:4) and Hp2 [5'-ATTACTGACGCTGATTGTGC] (SEQ ID NO:5) (Ho et al., J. Clin. Microbiol., 29:2543 (1991)), amplify a small unique region (from bases 744 to 853) of the H. pylori 16S rRNA gene. A PCR reaction, utilizing these primers results in a 109-bp amplicon. Primers 1281 [AACGCGCAAC] (SEQ ID NO:6) and 1290 [GTGGATGCGA] (SEQ ID NO:7) (Berg et al., Methods Mol. Cell. Biol., 5:13 (1994)) were used for the random amplified polymorphic DNA (RAPD) for H. pylori strain identification. These primers randomly amplify and produce a strain-specific banding pattern of DNA fragments. All primers were synthesized by Life Technologies (Rockville, MD).

Specificity of the PCR assay: Genus-specific primers (16S1-16S2) and species-specific primers (Hp1-Hp2) were used to amplify DNA extracted from pure cultures of H. pylori SS1, H. bilis, H. hepaticus, and H. muridarum. In addition, 5 to 10 μl of fecal DNA-extract was subjected to amplification reaction with the primer set Hp1-Hp2. Amplification reactions of 50 μl were performed as described (Ho et al., J. Clin. Microbiol., 29:2543 (1991)) with the following modifications: (I) annealing temperature was increased to 60°C and (II) 45 cycles were used. A sample of the PCR products was analyzed by electrophoresis on a 2% NuSieve agarose gel (FMC BioProducts, Rockland, ME).
A 109-bp PCR product was observed only with H. pylori DNA, and not with H. hepaticus, H. bilis, or H. muridarum when the Hp1-Hp2 species-specific primer set was used (Figure 1A, side A). In contrast, H. pylori, H. hepaticus, H. bilis, and H. muridarum demonstrated amplification of 375-bp products with the 16S1-16S2 genus-specific primer set (Figure 1A, side B). Thus, it is thought that the 16S1-16S2 primer set can be used generally to identify members of the Helicobacter genus, as opposed to the specificity of the Hp1-Hp2 primer set for H. pylori.

Specificity and selectivity of the PCR assay in stool containing genomic DNA from other Helicobacter species. DNA extracted from pure cultures of H. pylori SS1, H. bilis, H. hepaticus, and H. muridarum were mixed at concentration per Helicobacter species of 9 ng/μl. This mixture was serially diluted (10^3 to 10^6). The control DNA mixture contained only H. bilis, H. hepaticus, and H. muridarum. One fecal pellet from non-inoculated mice was added to 100 μl of each of the dilutions. After the addition of the proteinase-K reaction buffer and homogenization, DNA was extracted and amplified as described above using genus- and species-specific primers. Identical DNA samples minus fecal pellets were similarly analyzed as just described.

To demonstrate the specificity and selectivity of the PCR based H. pylori assay in stool contaminated with genomic DNA from other Helicobacter species, serially diluted genomic DNA mixtures contaminated with fecal material were amplified with genus- and species-specific primers. The results shown in Figure 1B, side C and Figure 1B, side D indicate that, in stool containing genomic DNA from three other Helicobacter species (lanes 2-4), the specificity and selectivity of PCR based H. pylori assay is unaltered. Lanes 5 and 11 show the results of the amplification of a DNA mixture that is minus H. pylori SS1 using species- and genus-specific primers, respectively. The absence of a PCR product in lane 5 versus lane 11 is consistent with the specificity and selectivity of the H. pylori species-specific primers, Hp1-Hp2.

Sensitivity of the PCR assay: To evaluate the detection limit of the assay, an overnight culture of H. pylori SS1 with a viable cell count of 10^9 CFU/ml was serially diluted to contain 10^8 to 10^1 CFU/ml. DNA was isolated from 100 μl of the serial dilutions (10^5 to 10^0 CFU/ml) using the method
described above for extracting DNA from mouse fecal pellets. The isolated DNA served as a template in PCR reactions using the Hp1-Hp2 primer set. To
examine whether fecal components interfered with the assay, a second 100 µl
sample of the serial dilutions (10^5 to 10^9 CFU/ml) was centrifuged, the
supernatant discarded, and one fecal pellet of non-inoculated mice added to
each tube. After suspension in 250 µl of the proteinase-K reaction buffer and
homogenization, DNA was extracted and tested as described above.

Sensitivity of the PCR assay in the absence of a fecal pellet: To
determine the detection limit of the assay, nucleic acid extracted from the serial
dilutions (10^5 to 10^9 CFU/ml) of H. pylori SS1 culture was amplified with
primers Hp1-Hp2. The results demonstrated that the sensitivity of the assay is 1
to 10 CFU/ml in aqueous solution (Figure 2A).

Sensitivity of PCR assay in the presence of fecal pellet: To examine
whether feces interfered with the assay, 100 µl of a 10-fold serial dilution (10^5
to 10^9 CFU/ml) of H. pylori SS1 culture was centrifuged, the supernatant
discarded, and one fecal pellet of non-inoculated mice was added to each tube.
After suspension, homogenization, and DNA extraction, the samples were
analyzed by PCR. H. pylori DNA was detected in serially diluted samples
(Figure 2B) as great as 10^8 (i.e., 10^2 bacterial CFU/ml). These results
demonstrated that in the presence of fecal components, the sensitivity of the
assay was reduced 10-fold. Thus, the sensitivity of this stool assay is estimated
at 10 to 100 bacterial cells per fecal pellet. The average weight of twenty fecal
pellets from C57BL/6 was 0.032 g; indicating that the sensitivity of the assay in
the presence of stool is 10-100 bacterial cells/0.032 g stool. It should be noted
that H. pylori infection in 0.025 g of human stool has been detected (data not
shown).

DNA fingerprinting

The random-amplified polymorphic DNA (RAPD) or arbitrarily primed
polymerase chain reaction (AP-PCR) DNA fingerprinting method was
performed as described (Berg et al., Methods Mol. Cell. Biol., 5:13 (1994)),
using primer 1281 or 1290. The amplicons were analyzed by electrophoresis on
a 2% NuSieve agarose gel. All amplification reactions were performed on an
automated thermocycler (GeneAmp PCR System 2400, Perkin-Elmer, Foster City, CA).

**DNA fingerprints of recovered bacteria:** An arbitrarily primed polymerase chain reaction DNA fingerprinting method was performed to determine whether the H. pylori strain recovered from cultures of gastric tissue of infected mice was the same as the H. pylori SS1 strain used for inoculation. The DNA samples were tested independently with the 10 nt primers 1281 and 1290. The DNA of bacteria recovered from gastric specimens obtained from mice produced a DNA fragment array pattern identical to that of DNA isolated from the H. pylori SS1 strain used for inoculation (Figure 4).

**PCR based detection of H. pylori in fecal pellets**

To determine the H. pylori status of mice non-invasively, fecal pellets of animals terminated at 6 months (N =15) were tested by PCR at 4 and 5 months post-inoculation, and animals terminated at 10 months (N =15) were tested at 9 months post-inoculation. In addition, fecal pellets were collected immediately before euthanasia and tested by PCR.

DNA (unquantitated) was extracted from supernatant fractions of fecal pellets of infected and non-infected mice. This DNA was subjected to PCR amplification, using primers Hp1-Hp2. The reaction produced 109 bp PCR fragments in most of the infected mice. Production of the 109 bp PCR fragment indicated H. pylori infection in 9 of 13 mice at 6 months, and 10 of 13 mice at 10 months. No H. pylori infection was detected in the 4 negative control mice (Table 1; Fig. 3).

**Detection of H. pylori at the time of necropsy**

**Histopathologic evaluation of gastric tissue:** Gastritis was mild at 6 months (Figure 5B) and it became severe at 10 months (Figure 5C). The presence of H. pylori was detected in Genta-stained slides of inoculated mice (13 of 13 mice at 6 months, and 12 of 13 mice at 10 months) (Figures 5B and 5C, insets; Table 1), and in 0 of 4 sham-inoculated mice (Figure 5A).

**Culture of H. pylori from gastric tissue:** Culture of gastric specimens on Campy Blood Agar plates and BLBB-GSSA plates resulted in growth of small
transparent colonies consistent with H. pylori in the majority of inoculated animals (10 of 13 mice, or 77% at 6 months; 11 of 13 mice, or 85% at 10 months), but in none of the 4 sham-inoculated mice (Table 1). Microscopy of positive cultures revealed motile, curved and spiral bacteria that tested positive for urease, catalase, oxidase and stained gram-negative (Munoz et al., Eur. J. Histochem., 42:297 (1998), Pinkard et al., J. Clin. Pathol., 39:112 (1986)). Counterstaining with carbolfuchsin, instead of safranin provided visualization of pink, curved organisms morphologically consistent with H. pylori. In addition, DNA extracted from these cultures and amplified with species-specific primers (Hp1-Hp2) produced a 109 bp PCR fragment, and RAPD fingerprinting of the same DNA with primers 1281 and 1290 demonstrated that the resulting profile was identical to that of the H. pylori SS1 strain used for inoculation (Figure 4). No growth was detected on BLBB-GSSA plates of the 4 negative control mice.

**PCR of gastric tissue:** Inoculated mice were positive by PCR (9 of 13 mice at 6 months, and 10 of 12 mice at 10 months), whereas 0 of 4 sham-inoculated mice tested positive (Table 1).

**PCR of fecal pellets:** At 6 months, fecal pellets were positive in 9 of 13 (69%) inoculated mice, and in 0 of 2 sham-inoculated control mice. At 10 months, 10 of 13 mice (77%) were positive (Figure 3; Table 1). The results indicate that 26 of 26 inoculated mice were positive by at least two of the three invasive tests performed directly on the stomach, and 4 of 4 sham-inoculated mice were negative by all three tests.

**Non-invasive detection of H. pylori in feces prior to euthanasia:** All 13 experimental mice that were terminated at 6-months tested positive at least once: 9 of 13 were positive at 3-months (69%); 9 of 13 were positive at 6-months (1-3 days before euthanasia), including three of the four mice that tested negative at 3-month. The fecal pellets collected on the day of euthanasia from the fourth mouse (that had tested negative at 3-month), also tested positive.

Similarly, the 13 animals euthanized at 10-months were tested 3 times by PCR of the stools (at 9-months, 9.5-months, and 10-months). All 13 animals tested positive at least once (8 positive 3 of 3 times, 3 positive 2 of 3 times, and 2 positive once). The fecal pellets collected at the time of euthanasia from one of
the mice that previously tested positive only once, also tested positive, while the other was negative. Accordingly, the sensitivity and the specificity of the stool PCR can be estimated at 100% if the test is performed at least four times. In addition, the fecal pellets were tested from a group of 5 mice (2 controls and 3 H. pylori infected) once daily for four days: 3 of 3 (100%) of the infected mice were positive 4 of the 4 times tested, and the two uninfected controls were negative. Duplicates of this four-day testing procedure, of the same group of mice, gave similar results with a range of 90 to 100% detection of H. pylori infection.

**Table 1. Percentage of *H. pylori* positivity by histology, culture, and PCR at the time of necropsy.**

<table>
<thead>
<tr>
<th></th>
<th>Inoculated Mice&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Sham-inoculated Mice&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6 months</td>
<td>10 months</td>
</tr>
<tr>
<td>Histology of gastric tissue</td>
<td>13/13 (100%)</td>
<td>12/13 (92%)</td>
</tr>
<tr>
<td>Culture of gastric tissue</td>
<td>10/13 (77%)</td>
<td>11/13 (85%)</td>
</tr>
<tr>
<td>PCR 1) gastric tissue</td>
<td>9/13 (69%)</td>
<td>10/12 (83%)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>2) fecal pellets</td>
<td>9/13 (69%)</td>
<td>10/13 (77%)</td>
</tr>
</tbody>
</table>

<sup>a</sup> All mice were positive by at least two of the three tests
<sup>b</sup> No tissue was available in one of the mice.
<sup>c</sup> Seven additional control animals remained negative up to 21 months,

**H. pylori status at the time of euthanasia**

Mice were fasted for 14 hours and euthanized by CO₂ asphyxiation and cervical dislocation. The stomach of each mouse was harvested and dissected along the greater curvature.

**Histology:** One half of the entire stomach was placed with mucosal side up in a Tissue-Tek Uni-Cassette (Sakura, Allegheny Healthcare Corp., McGraw Park, IL), immersed in neutral 10% buffered formalin, embedded in paraffin, sectioned, and stained with Hematoxylin and Eosin. Gastritis was evaluated on coded slides viewed at 100X. H. pylori colonization was assessed on coded.
Genta Robason-stained (Fox et al., *J. Clin. Microbiol.*, **33**:445 (1995)) slides viewed at 100X to 1000X.

The histological examination demonstrated colonization of the gastric corpus at a 6-month time point, and at a 10-month time point. Figure 5B (inset) shows minimal colonization of the surface epithelium while, figure 5C (inset) shows significant bacterial colonization. Infiltration by lymphocytes and some neutrophilic granulocytes tended to increase with time. There was mild to severe gastritis, but no ulcers were observed (Figures 5B and 5C).

**Culture of H. pylori:** Gastric tissue specimens (0.11 to 0.12 g) were taken from the antral and corpus regions, immediately immersed in 50 μl of BLBB containing 30% glycerol and 10% FCS, placed on dry ice and stored at -80°C. Harvested corpus and antral samples were homogenized with a sterile motorized pestle (Daigger, Lincolnshire, Illinois), and 100 μl of a 100-fold and 1000-fold dilutions of the homogenate were streaked on Campy Blood Agar plates (Remel, Lenexa, Kansas) and BLBB-GSSA plates containing 5 μg/ml of Amphotericin-B, 20 μg/ml of Bacitracin, 1.07 μg/ml of Nalidixic acid, 0.33 μg/ml of Polymyxin-B, and 10 μg/ml of Vancomycin (Glaxo Selective Supplement-A or GSSA) (McColm and Mobley, *Methods of Molecular Medicine*, vol. 8, Clayton, ed., Humana Press, Totowa, NJ (1997)). The plates were incubated under microaerophilic conditions at 37 °C and examined 3 to 4 days later. Pure colonies of *H. pylori* were obtained by sub-culturing the small transparent colonies, characteristic of *H. pylori*, on the BLBB-GSSA selective medium. These colonies were identified by a modified Gram stain (i.e., employing carbolfuchsin as counterstain), examined microscopically, and tested for urease, catalase, and oxidase activities (Munoz et al., *Eur. J. Histochem.*, **42**:297 (1998), Pinkard et al., *J. Clin. Pathol.*, **39**:112 (1986)). Finally, DNA extracted from cultures of the colonies was subjected to PCR analysis to test for *H. pylori* specific 16S rRNA (with Hp1-Hp2 primers) and for their RAPD-fingerprint profiles.

**PCR analysis:** DNA samples were prepared from stored gastric tissue specimen using the Qiagen Dneasy Kit protocol as described above. These DNA samples were tested for the presence of *H. pylori* by PCR with the Hp1-
Hp2 primer set and PCR conditions were as described above (Ho et al., J. Clin. Microbiol., 29:2543 (1991)).

Documents


Cutler and Toskes, Comparison of $^{13}$C-urea blood test to $^{13}$C-urea breath test for the diagnosis of Helicobacter pylori., Am. J. Gastroenterol., 94:959-961 (1999).


All publications, patents and patent applications cited herein are incorporated herein by reference. The foregoing specification has been described in relation to certain embodiments thereof, and many details have been set forth for purposes of illustration, however, it will be apparent to those skilled in the art that the invention is susceptible to additional embodiments and that certain of the details described herein may be varied considerably without departing from the basic principles of the invention.
**What is claimed is:**

1. A method to test for a microbe in a fecal sample obtained from an organism comprising:
   isolating amplifiable nucleic acid from the fecal sample; and
determining if the amplifiable nucleic acid includes a nucleic acid segment having a nucleotide sequence that is specific for the microbe.

2. The method of claim 1, wherein the microbe is a bacterium.

3. The method of claim 2, wherein the bacterium is a gram positive or gram negative bacterium.

4. The method of claim 2, wherein the bacterium is a Helicobacter bacterium.

5. The method of claim 4, wherein the bacterium is Helicobacter pylori.

6. The method of claim 1, wherein a nucleic acid amplification process is used to amplify the nucleic acid segment to produce an amplified product which indicates that the fecal sample contains a microbe.

7. The method of claim 6, wherein the nucleic acid amplification process is selected from the group consisting of polymerase chain reaction, ligase chain reaction, strand displacement activation, nucleic acid sequence-based amplification, branched DNA assay, invader assay, self-sustained sequence replication, ligation activated transcription, and repair chain reaction.

8. The method of claim 7, wherein a Helicobacter specific primer is used during the nucleic acid amplification process.

9. The method of claim 8, wherein the Helicobacter specific primer is selected from the group consisting of SEQ ID NOs: 1-7.
10. The method of claim 1, wherein the amplifiable nucleic acid is separated from the fecal sample through use of a silica support.

11. The method of claim 10, wherein the silica support is washed to reduce the presence of products that inhibit a polymerase chain reaction from the amplifiable nucleic acid.

12. The method of claim 11, wherein the nucleic acid is diluted prior to being used as a template in a polymerase chain reaction.

13. The method of claim 1, wherein the organism is a mammal.

14. The method of claim 13, wherein the mammal is a human.

15. The method of claim 13, wherein the organism is a mouse.

16. The method of claim 6, further comprising contacting the amplified product with a Taqman primer.

17. The method of claim 6, further comprising contacting the amplified product with a beacon or an amplifluor.

18. The method of claim 8, wherein the Helicobacter specific primer is a scorpion primer.

19. A method to determine if a population of a gastrointestinal microbe within an organism increased or decreased during a time period comprising
   a. determining an amount of nucleic acid that is specific for a microbe in a first fecal sample obtained from the organism at a first time point and in a second fecal sample obtained from the organism at a second time point; and
b. comparing the amount of nucleic acid that is specific for the microbe present in the first fecal sample to the amount of nucleic acid that is specific for the microbe present in the second fecal sample.

20. The method of claim 19, wherein the gastrointestinal microbe is a bacterium.

21. The method of claim 20, wherein the bacterium is a gram positive or gram negative bacterium.

22. The method of claim 20, wherein the bacterium is a Helicobacter bacterium.

23. The method of claim 22, wherein the bacterium is Helicobacter pylori.

24. The method of claim 19, wherein a nucleic acid amplification process is used to determine the relative amount of nucleic acid that is specific for the microbe in fecal samples obtained from the organism at the first time point and at the second time point.

25. The method of claim 24, wherein the nucleic acid amplification process is selected from the group consisting of polymerase chain reaction, ligase chain reaction, strand displacement activation, nucleic acid sequence-based amplification, branched DNA assay, invader assay, self-sustained sequence replication, ligation activated transcription, and repair chain reaction.

26. The method of claim 24, wherein a Helicobacter specific primer is used during the nucleic acid amplification process.

27. The method of claim 26, wherein the Helicobacter specific primer is selected from the group consisting of SEQ ID NOs: 1-7.
28. The method of claim 19, wherein the nucleic acid that is specific for the microbe is separated from the fecal sample through use of a silica support.

29. The method of claim 28, wherein the silica support is washed to reduce the presence of products that inhibit a polymerase chain reaction from the nucleic acid.

30. The method of claim 29, wherein the nucleic acid is diluted prior to being used as a template in a polymerase chain reaction.

31. The method of claim 19, wherein a therapeutic agent is administered to the organism.

32. The method of claim 31, wherein the therapeutic agent is a vaccine.

33. The method of claim 19, wherein the organism is a mammal.

34. The method of claim 33, wherein the mammal is a human.

35. A method to test efficacy of a vaccine against infection by Helicobacter bacteria comprising obtaining a fecal sample from a first vaccinated organism that was inoculated with the Helicobacter bacteria; and determining if the fecal sample contains an increased or decreased amount of Helicobacter specific nucleic acid when compared to an amount of Helicobacter specific nucleic acid contained within a fecal sample obtained from a non-vaccinated organism that was inoculated with the Helicobacter bacteria.

36. The method of claim 35, wherein the Helicobacter bacteria are Helicobacter pylori.
37. The method of claim 35, wherein a nucleic acid amplification process is used to determine if the fecal sample contains Helicobacter specific nucleic acid.

38. The method of claim 37, wherein the nucleic acid amplification process is selected from the group consisting of polymerase chain reaction, ligase chain reaction, strand displacement activation, nucleic acid sequence-based amplification, branched DNA assay, invader assay, self-sustained sequence replication, ligation activated transcription, and repair chain reaction.

39. The method of claim 37, wherein a Helicobacter specific primer is used during the nucleic acid amplification process.

40. The method of claim 39, wherein the Helicobacter specific primer is selected from the group consisting of SEQ ID NOs: 1-7.

41. The method of claim 35, wherein the Helicobacter specific nucleic acid is separated from the fecal sample through use of a silica support.

42. The method of claim 41, wherein the silica support is washed to reduce the presence of products that inhibit a polymerase chain reaction from the nucleic acid.

43. The method of claim 41, wherein the nucleic acid is diluted prior to being used as a template in a polymerase chain reaction.

44. The method of claim 35, wherein the organism is a mammal.

45. The method of claim 44, wherein the mammal is a human.

46. A kit comprising packaging material and a Helicobacter pylori specific primer.
47. The kit of claim 46, wherein the Helicobacter pylori specific primer is selected from the group consisting of SEQ ID NO: 1-7.

48. The kit of claim 46, wherein the Helicobacter specific primer is a molecular beacon or a scorpion primer.

49. The kit of claim 46, wherein the Helicobacter pylori specific primer comprises SEQ ID NO: 1.

50. The kit of claim 46, wherein the Helicobacter pylori specific primer has a nucleotide sequence corresponding to SEQ ID NO: 1.

51. The kit of claim 46, further comprising a support for isolation of nucleic acid.

52. The kit of claim 51, wherein the support is a silica-based support.

53. The kit of claim 51, further comprising a buffer.

54. The kit of claim 46, further comprising a thermostable polymerase.

55. An isolated nucleic acid segment comprising a nucleotide sequence as set forth in SEQ ID NO:1.

56. An isolated nucleic acid segment having a nucleotide sequence as set forth in SEQ ID NO:1.
SEQUENCE LISTING

<110>  Nyan, D.C.
      5    Coleman, W.G.
            Welch, A.R.

<120> Methods and compositions to detect microbes in fecal samples

10<130> 1662.011WO1

<160> 7

<170> FastSEQ for Windows Version 4.0
15
<210> 1
<211> 20
<212> DNA
<213> Helicobacter pylori
20
<400> 1
aacattactg acgctgattg 20

<210> 2
25<211> 18
<212> DNA
<213> Helicobacter pylori

<400> 2
30ctatgacggg tatccggc 18

<210> 3
<211> 19
<212> DNA
35<213> Helicobacter pylori

<400> 3
atccaccta cctctcaca 19

40<210> 4
<211> 20
<212> DNA
<213> Helicobacter pylori
ctggagagac atagcctcc 20

ctgattgtgc 20

aacgccgcaac 10

gtggatgcga 10