EVALUATION OF NON H. PYLORI SPIRAL ORGANISMS IN HUMAN GASTRIC BIOPSIES BY USING PCR AND MICROSCOPIC METHODS IN IRAN (FIRST REPORT)

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Abstract

Introduction and Objectives: The Discovery of *Helicobacter pylori* in 1982 increased interest in the range of other spiral bacteria that had been seen in Stomach (Marshall & Warren 1984).The power of technologies such as the polymerase chain reaction (PCR) with genus specific primers revealed that many of these bacteria belong to the genus Helicobacter. These non-pylori helicobacters are increasingly being found in human clinical specimens. Non-pylori Helicobacters are Gram-negative, motile, long, tightly coiled, Spiral bacteria ,with three to eight coils, that cause of some

gastric problems like gastritis, peptic ulceration and Mcosa-Associated Lymphoid Tissue (MALT) lynphoma in animals and humans.
Materials and Methods: Samples taken during endoscopy were analyzed by rapid urease test, PCR and light microscope(Giemsa and Gram staining). In this study 270 samples were collected from Patients with gastric disorders. Presence of Helicobacters confirmed by a positive urease test and Helicobacter genus specific PCR method utilized. DNA was prepared from biopsies using the Qiamp tissue kit (QIAGEN Inc., Valencia, Calif.) and frozen at −20°C (like gastric samples/biopsies). DNA samples that PCR positive were amplified with 16SrRNA gene primers against Helicobacter species.
Results: In gastric biopsy specimen's non-pylori helicobacter spp., have been observed. At the end of the study we found that 71% of urease tests, 0.37% of light microscopic studies (we observed some spiral gram negative bacteria with 2-7 coils) and 0.74% of PCR tests were positive. In analysis with PCR route 2 person (both of them were Male) were infected with H.heilmannii-like organisms(one of them kept a dog for 5 years as a pet).16S rRNA gene amplification was performed on 270 DNA samples and results were positive for H.heilmannii in two cases (275-bp), but negative for H.bizzozeronnii,H.felis and H. Salmonis.

Keywords: Non pylori helicobacters, gastric disorders, PCR

Introduction

Colonization by Helicobacter species is commonly noted in many mammals. These infections often remain unrecognized, but can cause severe health complications or more subtle host immune perturbations.Helicobacter Pylori is the primary cause of gastritis and peptic ulceration in humans and is a major risk factor for mucosa-associated lymphoid tissue (MALT) lymphoma and Adenocarcinoma. It was first reported in 1984 that gastric ulser disease in humans is caused by a bacterial infection (Marshall & Warren 1984).The discovery of *H. pylori* changed forever our perception of the stomach as a habitat for specialized bacteria. Besides the well-known gastric pathogen *Helicobacter pylori*, other *Helicobacter* species with spiral morphology have been detected in a minority of human patients who have undergone gastroscopy. In gastric biopsy specimens of a minority of patients with upper gastrointestinal symptoms, long, tightly coiled, spiral bacteria, ascribed to *non-pylori Helicobacter spp*., have been observed. Non-Helicobacter pylori helicobacters (NHPH) constitute a diverse group of bacterial species and very fastidious nature of these non-*Helicobacter pylori* helicobacters makes their *invitro* isolation difficult.

Microorganisms have been observed in stomach of animals and humans for more than 100 years. The first sighting has been credited to Rappin in 1881 who observed spiral-shaped bacteria in a dog's stomach. Bizzozero confirmed Rappin's discovery in 1892 and noted that these bacteria were associated with parietal cells of a dog stomach. Lim in 1920 added that some of the spiral-shaped organisms could be seen in the duodenum, close to the pyloric sphincter as well as in the pylorus, fundus, and the cardia. Non-pylori Helicobacter species are associated with a range of upper gastrointestinal symptoms, histologic, and endoscopic findings. The gastritis observed with H.heilmannii infection tends to be less severe than that due to H.pylori but infection has been found in association with duodenal ulceration, gastric ulceration, gastric carcinoma and mucosa associated lymphoid tissue (MALT) lymphoma (Morgner, et.al. 1995).

Although some data in the literature indicate that animals, including cats, dogs, and sheep, occasionally may be infected with *H. pylori* (Dore & Realdi 1999), (Fox, Dangler & Borkowski 1997), (Thompson & Gibson 1994), thereforer animals can play an important role in the transmission of this microorganism to humans.

Non-*H.pylori Helicobacter* infections of the human stomach are consistently accompanied by active chronic gastritis. These organisms have been designated `*Helicobacter heilmannii*'. However, sequencing of several genes detected in NHPH-infected tissues has shown that the `*H. heilmannii*' group comprises at least five different *Helicobacter* species, all of them known to colonize the stomach of animals (Weber & Schmittdie1962).“*H. heilmannii*” has also been associated with primary gastric low grade lymphoma in humans.

Recent investigations have indicated that *Helicobacter suis* is the most prevalent NHPH species in human. This species has only recently been isolated *in vitro* from porcine stomach mucosa. Other NHPH that colonize the human stomach are *Helicobacter felis, Helicobacter bizzozeronii, Helicobacter salomonis* and `*Candidatus* Helicobacter heilmannii'(Weber & Schmittdie1962).In numerous case reports of human gastric NHPH infections, no substantial information is available about the species status of the infecting strain, making it difficult to link the species with certain pathologies. It is proposed to use the term `gastric NHPH' to designate gastric spirals that are morphologically different from *H. pylori* when no identification is available at the species level. Some people infected with non-H.pylori helicobacters do not present obvious clinical signs (Mazzucchelli et al.1993).Terminal salic acid in the lipopolysaccharides (LPSs) of mucosal pathogens is an important virulence factor. Structural similarity between microbial and mammalian glycans is well-established

phenomenon in many Human mucosal pathogens (Weber & Schmittdie1962).

Clinical symptoms associated with non-*H. Pylori* helicobacters in humans can be characterized by atypical complaints such as acute or chronic epigastric pain and nausea. Other specific symptoms include hematemesis, recurrent dyspepsia, irregular defecation frequency and consistency, vomiting, heartburn, and dysphagia, often accompanied by a decreased appetite (Dieterich 1997), (Goddard, Rogan & Spiller 1997), (Heilmann & Borchard 1991), (Yang & Zhou 1998), (Kaklikkaya & Cobanoglu 2002), (Mention & Michaud1999), (Oliva & Perman 1993),(Schildt 2000),(Sykora and Hejda 2003), (Yoshimura & Isomoto 2002).

Of the known gastric *Helicobacter* spp, "*H heilmannii*" has the largest number of known mammalian hosts. *Helicobacter heilmannii* is the name proposed for a 4 to 10-μm-long, spiral-shaped, motile bacterium with three to eight coils, a wavelength of about 1 μm, up to 14, uni or bipolar flagella, and no periplasmic filaments (Aiba 1998), (Andersen and Norgaard 1996), (Andersen & Boye 1999), (Andersen & Norgaard 1996), (Jalava & Harrington2001), and (Svec & Kordas 2000). The bacterium was first described as "*Gastrospirillium hominis*" but was reclassified following 16S ribosomal DNA (rDNA) sequencing as "*H.heilmannii*". "*H. heilmannii*," like *H. pylori*, has been associated with gastritis, adenocarcinoma, and gastric mucosa-associated lymphoid tissue lymphoma. A diagnosis of humans infected with *H.heilmannii*, first observed and reported in three humans in 1987, has been made on morphological grounds by a variety of authors assessing human gastric biopsies. These gastric Helicobacter Like Organisms (HLOs) have commonly been observed microscopically in the stomachs of dogs, cats, cheetahs, swine, wild rats, various species of non-human primates, and in a small percentage of humans with gastritis (Aiba 1998), (Andersen and Norgaard 1996), (Ha¨nninen & Happonen1998) and (Haringsma & Mouwen 1992).Characterization of these bacteria has relied on 16S rRNA analysis because of the inability to grow the organisms on artificial media. Maintenance of bacteria in the laboratory, other than in a frozen state, has relied on preparation of these gastric spirals in the stomachs of mice.

The aim of the present study to assess the presence of animal Helicobacter species in humans with gastric disorders and infected with non pylori Helicobacter species as diagnosed by polymerase chain reaction (PCR) and light microscopic routs in Iran in order to better understand the possible zoonotic significance.

Materials And Methods

Infection in patients whit gastric disorders must confirm by a positive urease test. Definitive culture of H.heilmannii has not been achieved to date and diagnosis is usually made on the basis of its distinct Spiral morphology and genetics techniques such as PCR with specific primers are required for more definitive identification.

Sample collection

810 samples were collected from 270 patients with gastric disorders like Gastritis and ulcers. Gastric biopsy specimens of 270 patients with chronic or sever gastric disorders (110 women and 160 men) were collected with endoscopy method at the gastroentrology Department of some state and private hospitals and polyclinics and transported to the Microbiology laboratory under cool chain condition.

Various tests have been developed for the diagnosis of non pylori helicobacter Species infection. A small tissue sample (biopsy) was taken from the stomach during an endoscopy. The entire stomach was inspected for any abnormalities like erythema, erosion, ulcer, presence of sever or chronic gastritis, hypertrophy (edematous rugal folds) or atrophy (ability to observe the sub mucosal vessels).Biopsy forceps were used to obtain pinch biopsies from the abnormal regions. One biopsy specimen was taken and used for impression smear and light microscopic routs (gram and Giemsa stain of samples taken from the regions with abnormalities).The second biopsy, was taken from the same region for PCR analysis and frozen in sterile Phosphate-Buffered Saline (PBS) at -20°C and the third sample was used for rapid urease test.

R.U.T. (Rapid urease test): this test based on presence of copious quantities of urease (Eton et al., 1996; Lee, 1989) in Helicobacter spp. And hydrolyses of urea, the PH rises and color change from yellow to red occurs. Conversion to a pink-red color within 24h was considered as positive and the time was recorded (Shabestari & Jamshidi.n.d.).We performed the test at the time of gastroscopy. Samples were collected by the endoscopy method, and are placed into a medium containing urea (Urea agar medium) and an indicator such as phenol red. The urease produced by Helicobacter spp hydrolyzes urea to ammonia, which raises the PH of the medium, and changes the color of the specimen from yellow (NEGATIVE) to red or pink (POSITIVE).

Light Microscopy: spiral forms of non-pylori helicobacter species were found earlier in tissue sections by using light microscopy. At this study tissue samples were taken with biopsy forceps and studied after Gram and Giemsa staining by using light microscopic routs (× 100 magnifications).

PCR amplification of 16S rRNA gene:

For DNA extraction, biopsies were placed in a microcentrifuge tubes and frozen in PBS at −20°C. DNA was prepared from gastric biopsies by using the Qiamp tissue kit (QIAGEN Inc., Valencia, Calif.) according to the instructions of the manufacturer and DNA was stored frozen at −20°C.

As a result, Molecular techniques overcome some of the limitations of conventional culture under several clinical conditions.16S rRNA has highly conserved primer binding sites, Amplification of the universal 16S rRNA gene using PCR has improved the diagnostic yield of microbiological samples(Drancourt &Berger 2008,(Baltimore),p87:167–176), (Xu & Millar J. Appl. Microbiol. 94:197–206) and (Kolbert 1999 Jun). PCR was performed employing primers mentioned below(like HelF and HelR1) (Table 1) with 25 µl Taq Master Mix (QIAGEN Inc.) in a total volume of 50 µl (94°C for 10 min, followed by 30 cycles of 30 s at 94°C, 30 s at 58°C, and 30 s at 72°C) for the first stage by using termocyclere MJ Mini (personal thermocycler) BIO-RAD device. A 5-µl aliquot of the PCR product was transferred to a new tube containing the second-stage primers, HelF and HelR2 (25 pmol), and the other reagents, and cycle conditions were the same as those in the first round. PCR products were analyzed and visualized by electrophoresis on a 2% agarose gel.

Negative controls in which the DNA extract was replaced by sterile distilled water were included with each reaction and carried through as negative controls for the agarose gel DNA extraction process.

Table1: Sequences of specific primers and probes for PCR:

Primer or probe name	Gene	Nucleotide sequence	Specificity(ies)	Position
Hel F[c]	16S rRNA	5′-CGT-GGA-GGA-TGA-AGG-TTT-TA-3′	*Helicobacter* genus, PCR	402-421
Hel R1[c]	16S rRNA	5′-TAC-ACC-AAG-AAT-TCC-ACC-TA-3′	*Helicobacter* genus, PCR	667-686
Hel R2[c]	16S rRNA	5′-AAT-TCC-ACC-TAC-CTC-TCC-C-3′	*Helicobacter* genus, PCR	659-677
Hhe-3[c]	16S rRNA	5′-CCC-ACA-CTC-TAG-AAA-GAT-AG-3	"*H. heilmannii*"	642-661
Heibiz Sonde 7[c]	16S rRNA	5′-CCC-ACA-CTC-CAG-AGT-TGT-AG-3′	*H.felis,H.bizzozeronii, H.salomonis*	642-661
Heibiz Sonde 7C[c]	16S rRNA	5′-CCC-ACA-CTC-CAG-AGT-TGT-AG-3′	*H.felis,H.bizzozeronii, H.salomonis*	642-661

[c]Sequence from Trebesius et al. (Trebesius & Adler 2001).

Results:

In gastric biopsy specimen's non-pylori helicobacter spp., have been observed, Gram-staining indicated that these bacteria are Gram-negative. All samples that were positive in the urease test were colored red within 2-4 hours. Scanning light microscopic investigation revealed spiral-shaped Helicobacter-like organisms with 2-7 coils. We investigated PCR tests for various Helicobacter species including H. heilmannii, H. files, H. bizzozeroni and H. salomonis. At the end of the study we found that 71% of urease tests, 0.37% of light microscopic studies (we observed some spiral gram negative bacteria with 2-7 coils) and 0.74% of PCR tests were positive. In analysis with PCR route 2 person (both of them were Male) were infected with H.heilmannii-like organisms(one of them kept a dog for 5 years as a pet). We used species-specific primers mentioned above for detecting 16S rRNA gene on 270 biopsy samples and the results were positive for H.heilmannii in two cases (275-bp), but negative for H.bizzizeronnii,H.felis and H. Salmonis(table 2).

Table2: Infection status of non-pylori helicobacters infected patients

patients	***"Helicobacter heilmannii",***	***"Helicobacter bizzozeronii"***	***"Helicobacter felis"***	***"Helicobacter****. salomonis"*
110 women	-	-	-	-
160 men	2 (0.74%)	-	-	-

Helicobacter spp. was determined by use of species-specific PCR primers (see Materials and Methods).

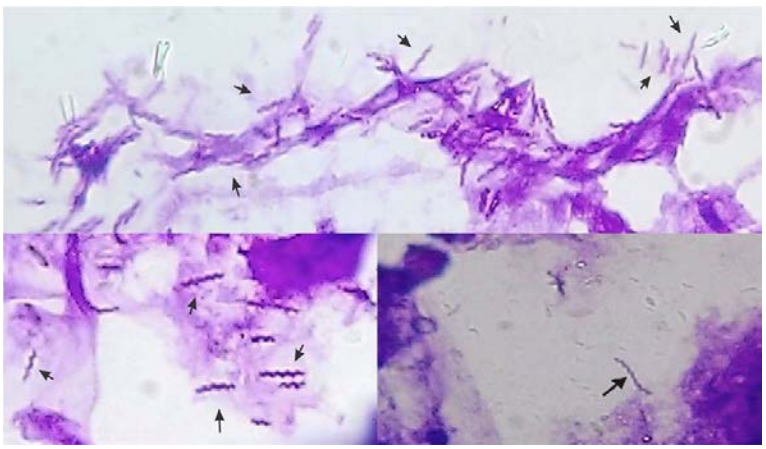

Fig. 1: Non-pylori Helicobacter like organisms in gastric sample, Giemsa staining (×1200 magnification)

Discussion

Some Helicobacter species usually associated with animals have also been detected in humans. These are sometimes referred to as "H. heilmannii," but this group probably includes several distinct species. The

stomachs of mammalian carnivores (e.g. cats, dogs or lions) are often naturally infected by non-pylori *Helicobacter* species, including *H. heilmannii, H. felis, H. bizzozeronii* and *H. salomonis,* which are very different from *H. pylori.*

There are clear indications that gastric helicobacters other than *H. pylori* can cause disease in humans (Trebesius & Haas 2001), (McNulty & Dent 2009, p.10). These tightly coiled microorganisms comprise at least five different *Helicobacter* species.

Diagnostic methods enabling the identification of these bacteria to the species level are needed to help clarify the epidemiology and pathology of these infections in humans. Studies demonstrates that Infection with *non-pylori Helicobacter spp* in humans is associated with some gastric disorders but only a small number of people with the infection develop peptic ulceration and (MALT) lynphoma. But it's not clear why some infected people develop ulcers and others don't. Our results confirm the specificity of PCR amplification of 16S rRNA gene for the identification of "*non-pylori Helicobacter spp*" and should be useful for discriminating these bacteria from other large spiral organisms in tissues from infected people.

The recent successes with in vitro isolation of these fastidious microorganisms from domestic animals open new perspectives for developing typing techniques that can be directly applied on gastric biopsies from humans. The availability of in vitro isolates also opens new perspectives for better understanding the pathogenesis of non- *pylori Helicobacter*-associated gastric pathology and for developing treatment and prevention measures.To gain more insights into the pathogenesis of these NHPH infections, it is important to obtain more information about the *Helicobacter* strains colonizing the human stomach. Therefore, an increased awareness among gastroenterologists and the application of more specific diagnostic methods are required to help clarify the epidemiology and pathology of these infections.

Estimates of the prevalence of human infection with non-H. pylori helicobacters range from 0.2– 6.0% have been reported from various countries (Baele et.al. 2009), (Smet et al. 2011, p.16:70–75). However, It seems that Iran has not a high infection rate,but further studies should be done statistically and epidemiologicaly with different genes and primers and also with more developed methods like DNA sequencing methods.Whereas, infection with these bacteria in humans is associated with gastritis and mucosa-associated lymphoid tissue lymphoma and is thought to be acquired by zoonotic transmission from dogs, cats or etc. According to Previous reports 10% of all NHPH infected persons develop peptic ulcers, 1–3% develop gastric cancer, and <0.1% develop another type of gastric tumor or mucosa-associated lymphoid tissue (MALT) lymphoma (Wroblewski et

al.2010, p. 23:713–739).We suggest that gastroenterologists must pay more attention in diagnosis and treatment of diseases or disorders that they can cause by Helicobacter species and they must note these microorganisms in their decision and treatment procedure.

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