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Non *H. pylori* helicobacter identified as *H. heilmannii* in gastric biopsy samples in humans with gastric disorders by PCR and microscopic methods in Iran (First report)

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ABSTRACT

The Discovery of Helicobacter pylori in 1982 increased interest in the range of other spiral bacteria that had been seen in Stomach.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. Samples taken during endoscopy were analyzed by rapid urease test, PCR and light microscope(Giemsa and Gram staining). In this study 810 biopsy samples from 270 patients with gastric disorders were collected. 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 were amplified with 16SrRNA gene primers against Helicobacter species. 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.

Key words: non pylori helicobacters, H.heilmannii, Gastric disorders, PCR

INTRODUCTION

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). 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. 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.

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). Researches indicate that animals like cats occasionally can be infected with H. Pylori, 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 can colonize in the stomach of animals and “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.Other NHPH that colonize the human stomach are Helicobacter felis, Helicobacter bizzozeronii, Helicobacter salomonis and `Candidatus Helicobacter heilmannii'. 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 (Mazzucchelliet al.1993).

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 appetit (Sykora and Hejda 2003) and (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, uni or bipolar flagella, and no periplasmic filaments (Jalava & Harrington2001). 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. First report of human infection with H.heilmannii, reported in three humans in 1987 and it 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 (Haringsma & Mouwen 1992). 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

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). 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):
Urease test performed during the time of gastroscopy. Conversion to a pink-red color within 24h was considered as positive and the time was recorded (Shabestari & Jamshidi.n.d.). Samples were 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:
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. 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). PCR was performed employing primers mentioned below (like HelF and HelR1) (Table 1) with 25 µl Taq Master Mix (QIAGEN Inc.) with using termocyclere MJ Mini BIO-RAD device.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.

TABLE 1: 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

Sequence from Trebesius et al. (Trebesius & Adler 2001)

RESULTS

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).

TABLE 2: 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).

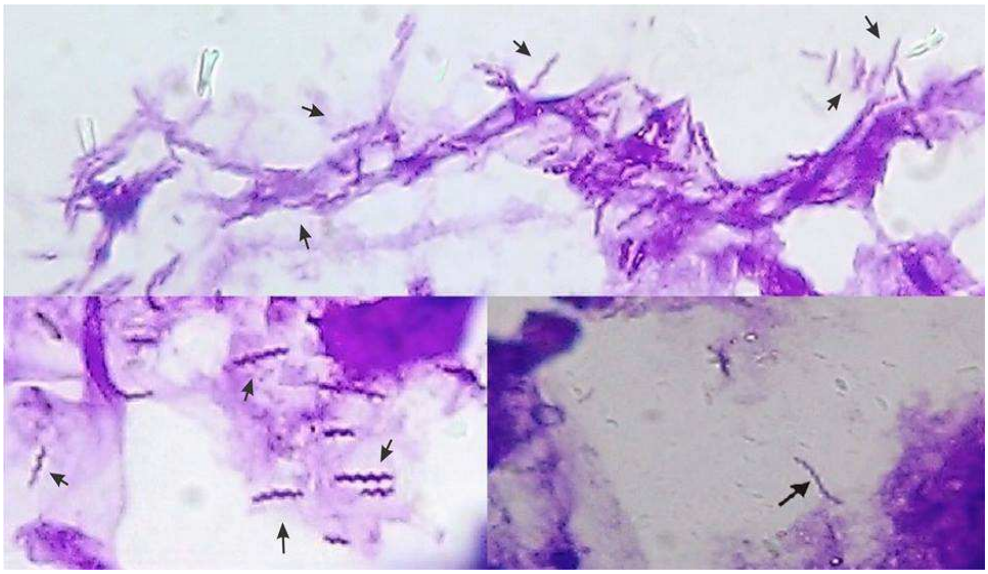

Figure 1: Non-pylori Helicobacter like organisms in gastric sample, Giemsa staining (×1200 magnification)

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 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 (McNulty & Dent 2009). These tightly coiled microorganisms comprise at least five different Helicobacter species(mentioned above).

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.

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). 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. 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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