Use of Polymerase Chain Reaction and Enzymatic Cleavage in the Identification of *Helicobacter* spp. in Gastric Mucosa of Human Beings from North Paraná, Brazil

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Helicobacter pylori *is the most common gastric bacteria of human beings. Animal-borne helicobacter have been associated with gastritis, ulceration, and gastric mucosa-associated lymphoid-tissue lymphoma in people. We attempted to identify the species of* Helicobacter *spp. that infect human beings in north Paraná, Brazil. Samples of gastric mucosa from 38 dyspeptic patients were analyzed by optic microscopy on silver stained slides, polimerase chain reaction (PCR), and enzymatic cleavage. Genus and species-specific primers to* H. pylori, H. heilmannii, H. felis, *and consensual primers to* H. bizzozeronii *or* H. salomonis *were used. The PCR products were submitted to enzymatic cleavage by VspI* (Helicobacter *spp. product) and* Hin*fI (species products) enzymes. Thirty-two out of 38 patients evaluated had 3.2 to 5 µm long bacteria that resembled* H. pylori *in Warthin-Starry stained slides and were positive to the genus* Helicobacter *by PCR. In 30 of these patients the bacteria were identified as* H. pylori. *Two samples positive by silver stain were negative to all species tested by PCR. None of the 38 samples was positive to animal-origin helicobacter species. These results show that PCR and enzymatic restriction are practical methods to identify the species of helicobacters present in gastric mucosa of human beings. People in north Paraná appear to be infected mostly with* H. pylori.

Key words: *Helicobacter* spp. - dyspeptic patients - diagnosis - polymerase chain reaction - Paraná - Brazil

Helicobacter pylori is the most prevalent helicobacter species in human beings. Helicobacter species, usually found in dogs, cats, and pigs, have been associated in different parts of the world with gastric inflammation, ulcers and neoplasia, especially mucosal associated-lymphoid tissue lymphoma (MALT) in humans (Morris et al. 1990, Solnick et al. 1994, Dieterich et al. 1998, Morgner et al. 2000). Dogs, cats, and pigs may have been the source of infection to humans (Lavelle et al. 1994, Stolte et al. 1994, Dieterich et al. 1998, Meining et al. 1998).

Infection rates in humans for helicobacters other than *H. pylori* are usually low, ranging from 0.01% in Italy (Foschini et al. 1999) to 6.2% in Thailand (Yali et al. 1998), a developing country. The prevalence of the less common helicobacters in human gastric mucosa is unknown. *H. heilmannii* type 1 apparently is the most prevalent bacteria (78.5%), moreover there are infections by *H. salomonis* (2.4%) and non-identified species (Trebesius et al. 2001).

H. heilmannii may cause MALT lymphoma, because spontaneous regression of gastric lymphoma type MALT occurs after its eradication (Regimbeau et al. 1997, Morgner et al. 2000). It has been suggested that the best therapeutic approach to eradicate *H. heilmannii* may not be the same used to eradicate *H. pylori* in humans (Foschini et al. 1999).

Tightly spiraled bacteria have been found occasionally in the gastric mucosa of dyspeptic patients in Brazil (Queiroz et al. 1990, Nogueira et al. 1993), but the species or the source of infection were not determined.

In a swine population in the State of Minas Gerais, *H. heilmannii* was present in 77% (54 out 70) of the stomachs (Queiroz et al. 1996). In the northern region of the State of Paraná, 64% (32 out 50) of the cats were infected by *H. heilmannii*, whereas *H. felis* was identified in 62% (31 out 50) of the cats and 51% (36 out 70) of the dogs (Camargo 2002).

Since the species that infect humans in our region have not been determined, we attempted to verify the adequacy of PCR associated to enzymatic restriction in order to determine which *Helicobacter* species inhabit the gastric mucosa in dyspeptic patients from the north of Paraná, Brazil.

MATERIALS AND METHODS

Patients - Samples from gastric mucosa were collected from 38 dyspeptic patients admitted in the Gastroenterology section, Hospital Universitário, Universidade Estadual de Londrina, in Londrina, PR, Brazil. Men and women, aged from 26 to 89 years, were included in the study. Only patients with clinical indication for upper endoscopy and mucosa collection were admitted in the study.

Mucosa biopsy samples from the stomach body and antrum were obtained for histological and molecular biology analysis.

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Morphological analysis of bacteria - Mucosal fragments from each patient were fixed in 10% phosphate-buffered formalin. Sections of paraffin-embedded specimens were applied onto glass slides, and stained by the Warthin-Starry technique (Michalany 1980). The slides were scanned for presence of bacteria and morphological analysis.

DNA extraction - Mucosal specimens from each patient were sealed into Eppendorf vials and mixed to 50 µl of proteinase K (20 mg/ml) and 200 µl of lyses buffer pH 8 (100 mM of NaCl, 100 mM of EDTA and 0.5% of dodecyl sodium sulphate), agitated for 30 sec and then incubated at 56ºC for 3 h. DNA was extracted using phenol/chloroform (Sambrook et al. 1989), followed by guanidine/isothiocyanate silica (Boom et al. 1990).

PCR amplification - PCR was performed in a final volumes of 50 µl solutions composed of 10 µl of DNA mix [(4 µl of extraction product, 4 µl of ultra pure water, and 20 pM of each of the oligonucleotide primers (Table I)], plus 40 µl of *Taq* PCR mix containing 23.5 µl of ultra pure water, 1.6 mM of dNTP, 4 µl of PCR buffer (20 mM of Tris HCl pH 8.4 and 50 mM of KCl), 2.5 U of *Taq* DNA polymerase recombinant, and 1.5 mM of $MgCl_2$. Amplification were performed in a thermocycler (PTC-100™ - MJ Research Inc.) under PCR conditions described in Table II.

DNA of *H. pylori* and *H. felis* standard strains were used as positive controls. Positive controls for the other species were developed by presumptive identification by transmission electron microscopy of bacteria present in gastric mucosa from dogs and cats. Presumptive identification was confirmed by PCR using species-specific primers and enzymatic restriction.

All gastric mucosal samples, including those non-reactive to the genus primer, were tested for the species *H. pylori*, *H. heilmannii*, *H. felis*, and *H. bizzozeronii* or *H. salomonis*.

PCR products were electrophoresed in 1.5% (w/v) agarose gel with 0.3% ethidium bromide in a 10% Tris-Borate-EDTA buffer (TBE). Readings were performed in UV light illumination. Photographic registration was done using a digital Kodak system (Kodak EDAS 290™).

DNA enzymatic restriction - Products amplified with genus primers were cleaved by *Vsp*I enzyme (Germani et al. 1997). *H. pylori* amplified products were cleaved by *Hin*fI (Clayton et al. 1992). DNA restriction digestion and electrophoresis were performed by standard procedures (Sambrook et al. 1989).

The sequences of amplified products from *H. heilmannii*, *H. bizzozeronii* or *H. salomonis* and *H. felis* primers were estimated based on sequences present in the GenBank. The length of fragments generated with *Hin*fI cleavage was established using the Gene Run™ software.

RESULTS

In 32 out of 38 patients small slightly curved or S-shaped bacteria with size ranging from 3.2 to 5 µm in length were visualized on Warthin-Starry stained slides. The bacteria were distributed on mucosal surface and in the glandular pits. The same 32 patients were positive by PCR for *Helicobacter* spp. (Fig. 1).

TABLE I

Oligonucleotide primers used in this study, reference source, primer sequence and length of amplified fragment (in base pairs)

Genes target	Reference	Primer sequence (5' ——— 3')	Amplified fragment
16S rRNA genes of *Helicobacter* spp.	Germani et al. (1997)	(f) AAC GAT GAA GCT TCT AGC TTG CTA (r) GTG CTT ATT CGT GAG ATA CCG TCA T	399 bp
ureB genes of *H. bizzozeronii* or *H. salomonis*	Neiger et al. (1999)	(f) ACT AGG CGA TAC CAA CTT GAT TT (r) TTC TTC AGC TGC GCG GAG CAT GC	499 bp
ureA and B genes of *H. felis*	Germani et al. (1997)	(f) GTG AAG CGA CTA AAG ATA AAC AAT (r) GCA CCA AAT CTA ATT CAT AAG AGC	241 bp
ureB genes of *H. heilmannii*	Neiger et al. (1998)	(f) GGG CGA TAA AGT GCG CTT G (r) CTG GTC AAT GAG AGC AGG	580 bp
ureB genes *H. pylori*	Clayton et al. (1992)	(f) GCC AAT GGT AAA TTA GTT (r) CTC CTT AAT TGT TTT TAC	411 bp

(f): foward sequence; (r): reverse sequence; bp: base pairs

TABLE II

Conditions of polymerase chain reaction reactions used in this study

Species tested	Initial denaturation step	Temperature of denaturation, aneling and extension	Number of cycles and duration	Final extension step
Helicobacter spp.	95ºC for 5 mim	95ºC, 65ºC and 72ºC	40 for 1 mim	72ºC for 7 min
H. bizzozeronii or *H. salomonis* and *H. felis*	94ºC for 5 mim	94ºC, 62ºC and 72ºC	40 for 1 min	72ºC for 10 min
H. heilmannii	94ºC for 3 mim	94ºC, 57ºC and 72ºC	35 for 1 mim	72ºC for 5 min
H. pylori	95ºC for 5 mim	94ºC, 45ºC and 72ºC	40 for 1 min	72ºC for 10 min

Of the 32 patients with *Helicobacter* spp., 30 were identified by PCR as having *H. pylori* (Fig. 2). The two remaining patients were negative to all species tested.

Cleavage of the products amplified by genus specific primer (399 bp) using *Vsp*I enzyme resulted in fragments of 295 bp and 104 bp in length. Cleavage using *Hin*fI enzyme resulted in fragments with 277 bp and 134 bp of the *H. pylori* (411 bp) amplified products, 408 and 172 bp for *H. heilmannii*; 100 bp for *H. felis*, and 395 and 100 bp length for *H. bizzozeronii* or *H. salomonis* (Fig. 3).

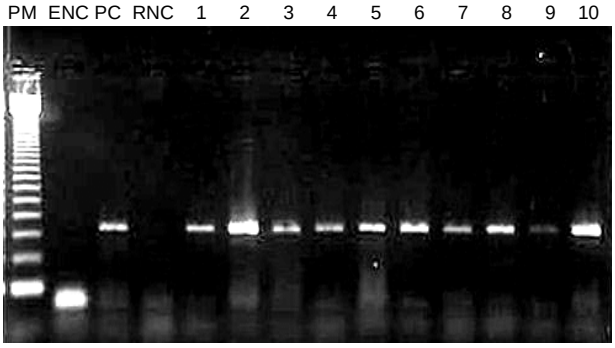

Fig. 1: bands with approximately 399 bp generated in polymerase chain reaction by *Helicobacter* spp. primers in agarose gel (1.5% w/v); PM: 123 bp molecular weight marker; ENC: extraction negative control; RNC: reaction negative control; PC: positive control; 1 to 10 patient samples

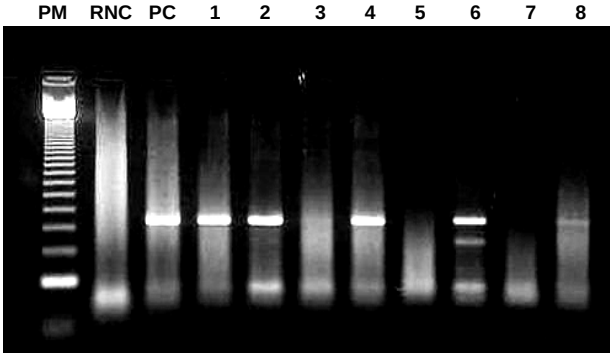

Fig. 2: bands with approximately 411 bp generated in polymerase chain reaction by *Helicobacter pylori* primers in agarose gel (1.5% w/v); PM:123 bp molecular weight marker; RNC: reaction negative control; PC: positive control; 1 to 8 patient samples

DISCUSSION

The bacteria visualized with the Warthin-Starry staining showed size and morphology compatible with *H. pylori*. When compared to the bacteria found on slides of gastric mucosa from dogs and cats, stained by the same technique, remarkable differences in both morphology and size could be observed. *H. pylori* can be differentiated from animal-infecting species by optical microscopy if adequate staining is used (Queiroz et al. 1990, Lavelle et al. 1994, Goddard et al. 1997, Foschini et al. 1999).

The *Helicobacter* genus-specific primers to PCR amplification of 16S rRNA genes, to *H. felis* urea A and urea B genes (Germani et al. 1997), and urea A to *H. pylori* (Clayton et al. 1992), and urea B genes to *H. heilmannii* and *H. bizzozeronii* or *H. salominis* (Neiger et al. 1998, 1999) amplified products of expected sizes.

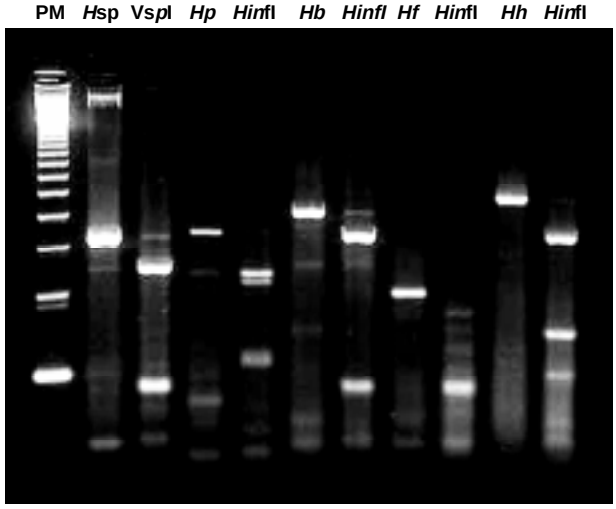

Fig. 3: pattern generated by cleavage of polymerase chain reaction products with *Vsp*I enzyme and *Hin*fI enzyme, and length of restriction product in base pairs. Agarose gel (2.5% w/v); PM: 123 bp molecular weight marker; *H*sp: *Helicobacter* spp. amplified material without restriction; *Vsp*I: restrict by *Vsp*I enzyme; *Hp*: *H. pylori* amplified material without restriction; *Hin*fI: restrict by *Hin*fI enzyme; *Hb*: *H. bizzozeronii* or *H. salomonis* amplified material without restriction; *Hf*: *H. felis* amplified material without restriction; *Hh*: *H. heilmannii* amplified material without restriction

Enzyme cleavage of the PCR-generated products with *Vsp*I enzyme (Germani et al. 1997) and *Hin*fI enzyme (Clayton et al. 1992) was a rapid and practical method to confirm that the genetic material amplified was from *Helicobacter* spp. and *H. pylori.*

The restriction of the PCR-generated products with primers to the species *H. bizzozeronii* or *H. salomonis* and *H. heilmannii* generated fragments of expected length. In the *H. felis* amplified material however, two bands of 100 bp were superposed and the other expected band (41 bp) could not be seen in agarose gel due to its small size. The use of polyacrylamide gel might have allowed visualization of the smaller bands.

The association of specific primers with enzymatic cleavage allowed species identification in a large number of samples, showing it to be a practical and reliable tool for routine laboratory use.

Samples from two patients were positive by PCR for the *Helicobacter* genus, but negative to all tested species. Technical errors and defective reaction with the primers are unlikely, based on the presence of positive controls. These patients may have hosted a *Helicobacter* species different from those tested.

The low infection rate of human beings with animal specific *Helicobacter* spp. probably results from a host-specificity among these bacteria. Nevertheless small infection rates with animal-origin helicobacter were reported in Europe (Foschini et al. 1999) higher rates (6.2%) were reported in Thailand (Yali et al. 1998).

In the State of Minas Gerais, Brazil, one out of 315 dyspeptic patients (0.4%) was infected with both, *H. pylori* and "*Gastrospirillum hominis*" (Queiroz et al. 1990), an animal-origin helicobacter. Also in Minas Gerais 2.5%

of low socioeconomic level patients (one out of 40) with gastric cancer also had mixed infection (Nogueira et al. 1993). All samples tested with species-specific primers for helicobacters other than *H. pylori* yielded, negative results despite the fact that these patients have low socioeconomic level, and live in a region with a high incidence of *H. felis* and *H. heilmannii* in dogs and cats (Camargo 2002). Lack of close contact with animals could be a plausible hypothesis to explain the absence of animal-origin helicobacters in the patients.

Pets may still be a source of infection to humans. Organisms similar to ones found in humans with gastric diseases were also found in their pets (Lavelle et al. 1994, Dieterich et al. 1998). In one case clinical remission of gastric signs in a human patient was only achieved after eradicating the bacteria from both patient and his two pet dogs (Thomson et al. 1994).

Despite questions about the zoonotic potential of these microorganisms, species identification within the *Helicobacter* genus is important. There is an association between infection by *H. heilmannii* and gastric lymphoma type MALT. Remission is achieved when the bacteria are eradicated (Regimbeau et al. 1997, Morgner et al. 2000). Gastric ulceration related with *H. heilmannii* improved after eradication of these bacteria (Goddard et al. 1997).

If it is necessary to eradicate the bacteria in order to achieve cure in human patients, the precise species identification and known animal sources of infection become even more important. Therapeutic strategies designed to eradicate animal-born helicobacter may differ from the ones used to eradicate *H. pylori* in human patients.

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REFERENCES

Boom R, Sol CJA, Salimans MMM, Jansen CL, Van Dillen WPME, Van Der Noordaa J 1990. Rapid and simple method for purification of nucleic acids. *J Clin Microbiol 28*: 495-503.

Camargo PL 2002. *Identificação das Espécies de Helicobacter spp. Presentes na Mucosa Gástrica de Cães, Gatos e Seres Humanos, e sua Correlação com as Alterações da Mucosa Gástrica*, PhD Thesis, Universidade de São Paulo, São Paulo, 93 pp.

Clayton CL, Kleanthous H, Coates PJ, Morgan DD, Tabaqchali S 1992. Sensitive detection of *Helicobacter pylori* by using polymerase chain reaction. *J Clin Microbiol 30*: 192-200.

Dieterich C, Wiesel P, Neiger R, Blum A, Corthésy-Theulaz I 1998. Presence of multiple "*Helicobacter heilmannii*" strains in an individual suffering from ulcers and in his two cats. *J Clin Microbiol 36*: 1366-1370.

Foschini MP, Pieri F, Cerasoli S, Accardo P, Formica G, Biasiucci A, Donzelli C, Villanacci V 1999. *Helicobacter heilmannii*: studio anatomo-clinico di 14 nuovi casi. *Pathologica 91*: 18-24.

Germani Y, Dauga C, Duval P, Huerre M, Levy M, Pialoux G, Sansonetti P, Grimont PAD 1997. Strategy for the detection of helicobacter species by amplification of 16S rRNA genes and identification of *H. felis* in a human gastric biopsy. *Res Microbiol 148*: 315-326.

Goddard A, Logan RPH, Athernon JC, Jenkins D, Spiller RC 1997. Healing of duodenal ulcer after eradication of *Helicobacter heilmannii*. *Lancet 349*: 1815-1816.

Lavelle JP, Landas S, Mitros FA, Conklin JL 1994. Acute gastritis associated with spiral organisms from cats. *Dig Dis Sci 39*: 744-750.

Mening A, Kroher G, Stolte M 1998. Animal reservoirs in the transmission of *Helicobacter heilmannii*. Results of a questionnaire-based study. *Scand J Gastroenterol 33*: 795-798.

Michalany J 1980. *Técnica Histológica em Anatomia Patológica*, Editora Pedagógica e Universitária Ltda., São Paulo, 273 pp.

Morgner A, Lehn N, Andersen LP, Thiede C, Mads B, Benndesen M, Trebesius K, Neubauer B, Neubauer A, Stolte M, Bayerdörffer E 2000. *Helicobacter heilmannii*-associated primary gastric low-grade MALT lymphoma: complete remission after curing the infection. *Gastroenterology 118*: 821-828.

Morris A, Ali MR, Thomsen L, Hollis B 1990. Tightly spiral shaped bacteria in the human stomach: another cause of active chronic gastritis? *Gut 31*: 139-143.

Neiger R, Dieterich C, Burnens A, Waldvogel A, Corthésy-Theulaz I, Halter F, Lautergurg B, Schmassmann A 1998. Detection and prevalence of helicobacter infection in pet cats. *J Clin Microbiol 36*: 634-637.

Neiger R, Tschudi ME, Burnens A, Göke B, Schmassmann A 1999. Diagnosis and identification of gastric *Helicobacter* species by polymerase chain reaction in dogs. *Microbiol Ecol Heal Dis 11*: 234-240.

Nogueira, AMMF, Ribeiro GM, Rodrigues MAG, Queiroz DMM, Mendes EN, Rocha GA, Barbosa AJA 1993. Prevalence of *Helicobacter pylori* in Brazilian patientes with gastric carcinoma. *Am J Clin Pathol 100*: 236-239.

Queiroz DM, Rocha GA, Mendes EN, DeMoura SB, Oliveira AM, Miranda D 1996. Association between *Helicobacter* and gastric ulcer disease of the pars esophagea in swine. *Gastroenterology 111*: 19-27.

Queiroz DMM, Cabral MMDA, Nogueira AMMF, Barbosa AJA, Rocha GA, Mendes EN 1990. Mixed gastric infection by *Gastrospirillum hominis* and *Helicobacter pylori*. *The Lancet 336*: 507-508.

Regimbeau C, Karsenti D, Durand V, D'Alteroche L, Copie-Bergman C, Metman EH, Machet MC 1997. Lymphome gastric de bas grade du MALT et *Helicobacter heilmannii* (*Gastrospirillum hominis*). *Gastroenterol Clin Biol 22*: 720-723.

Sambrook, J, Fritsch EF, Maniatis T. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor, New York, 1659 pp.

Solnick JV, O'Rouke J, Lee A, Paster BJ, Dewhirst FE, Tompkins LS 1994. An uncultured gastric spiral organism is a newly identified *Helicobacter* in humans. *J Infec Dis 168*: 379-385.

Stolte M, Wellens E, Bethke B, Ritter M, Eidt H 1994. *Helicobacter heilmannii* (formerly *Gastrospirillum hominis*) gastritis: an infection transmitted by animals? *Scand J Gastroenterol 29*: 1061-1064.

Thomsom MA, Storey P, Greeer R, Cleghorn GJ 1994. Canine-human transmission of *Gastropirillum hominis* (note). *Lancet 343*: 1605-1607.

Trebesius K, Adler K, Vieth M, Stolte M, Haas R 2001. Specific detection and prevalence of *Helicobacter heilmannii*-like organisms in the human gastric mucosa by fluorescent *in situ* hybridization and partial 16S ribossomal DNA sequencing. *J Clin Microbiol 39*: 1510-1516.

Yali Z, Yamada N, Wen M, Matsuhisa T, Miki M 1998. *Gastrospirillum hominis* and *Helicobacter pylori* infection in Thai individuals: comparison of histopathological changes of gastric mucosa. *Pathol Int 48*: 507-511.