Immunodetection of *Helicobacter* sp. and the associated expression of ABO blood group antigens in the gastric mucosa of captive and free-living New World primates in the Amazon Region

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The histo-blood group ABH antigens were first described in humans. These antigens are only present on erythrocytes from great apes and humans, while in more primitive animals they are found in tissues and body fluids. The ABH antigens are mainly distributed in tissues exposed to the external environment and potentially serve as ligands for pathogens or inhibitors of tissue connections. The objective of this paper was two-fold: (i) to determine the presence of Helicobacter *sp. in the gastric mucosa of 16 captive and 24 free-living New World monkeys and (ii) to evaluate the presence of histopathological alterations related to bacterial infection and the associated expression of ABH antigens in the tissue. Stomach tissues from 13 species of monkey were assessed using haematoxylin-eosin and modified Gram staining (Hucker) methods. An immunohistochemical analysis of the tissue revealed the presence of infectious bacteria that were characteristic of the genus* Helicobacter *sp. The results demonstrate that various species of monkey might be naturally infected with the* Helicobacter *sp. and that there is an increased susceptibility to infection. This study serves as a comparative analysis of infection between human and non-human primates and indicates the presence of a new species of* Helicobacter.

Key words: New World primates - *Helicobacter* sp. - ABO blood group system - immunohistochemistry

Helicobacter are helicoidal, flagellated, Gram-negative and microaerophilic bacteria that include a large number of species isolated from the gastrointestinal tracts of humans and other animal species, such as Old and New World primates (Dubois et al. 1994, Lundström et al. 2001, Tamashiro et al. 2005).

Baskerville and Newell (1988) have described the clinical association of chronic gastritis due to the natural infection of Old World primates (*Macaca mulatta*) by the bacterial species *Helicobacter pylori*. Other studies have described how rhesus monkeys also develop gastric pathologies, such as peptic ulcers and even stomach cancer, when infected by *H. pylori* (Dubois et al. 1991, 1994, 1999). Therefore, these animals have been used as models for comparative studies with humans. The induced infection of the New World *Saimiri* sp. resulted in a slight and temporary inflammation of the mucosa in a great majority of animals (Stadtländer et al. 1998). Thus, the results from studies focused on understanding *Helicobacter* spp infections and associated diseases in various hosts might help to increase our understanding of pathogenic mechanisms (Atherton 2005).

A new species of *Helicobacter* was isolated and associated with chronic colitis in the New World monkey species *Saguinus oedipus* (Saunders et al. 1999). In 2005, Mello et al. (2005) described the presence of these bacteria in the gastric environment of marmosets (*Callithrix jacchus*) and suggested that a natural bacterial colonisation could occur among these primates. More recently, a new species of *Helicobacter* (*Helicobacter callitrichis*) was isolated from *C. jacchus* (Tamashiro et al. 2005).

Additionally, polymorphism in the ABO blood groups of humans and other primates, determined by the expression of the A, B or H(O) antigens, is made up of fucosylated and terminal oligosaccharides linked to proteins and lipids, whose main function responsible for and capable of promoting their conservation throughout evolution still remains unknown (Fox et al. 2008). These antigens are distributed in tissues and are primarily localised in epithelial cells that function as a barrier to the external environment; hence, it is believed that these antigens could either serve as ligands to specific pathogens or inhibit pathogenic interactions at the cell surface (Henry 2001).

The direct interactions of *H. pylori* with ABH antigen structures initially demonstrated that individuals expressing fucosylated antigens (H and Lewis b) were more susceptible to disease because the microorganism utilises these epitopes to adhere to host cells (Borén et al. 1993). The presence of such receptors has been investigated in the gastric mucins of rhesus monkeys (Lindén et al. 2004) and the results are consistent with previous evidence.

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Studies in human adults and children in Brazil (Martins et al. 2006, Rodrigues et al. 2007) and other countries (Brigić et al. 2002, Kanbay et al. 2005) indicate an association of ABO blood types with *H. pylori* infection. Individuals with O and Lewis b phenotypes demonstrated an increased susceptibility to infection and colonisation, which resulted in the development of gastric pathologies.

Therefore, it is important to understand the epidemiology and zoonotic potential of helicobacteriosis and to identify new *Helicobacter* spp from non-human primates because their susceptibility to many species-specific pathogens is similar to humans and, thus, they serve as valuable models for studying human infectious diseases (Bennett et al. 1998, Tamashiro et al. 2005).

In this study, we identified the presence of *Helicobacter* sp. in different species of captive and free-living New World primates and examined the occurrence of histopathological changes related to this infection. Moreover, we examined the expression of these antigens in the gastric mucosa as markers for genetic predisposition to bacterial infection.

MATERIALS AND METHODS

Animals - Wild monkeys were obtained from the following distinct geographical areas in the Amazon Region, Brazil: Amazon National Park, a municipality of Itaituba located in the southwestern area of the state of Pará (PA), Mamirauá Sustainable Development Reserve, state of Amazonas (AM), located in the middle of the Solimões River region, Amanã Sustainable Development Reserve, Central Amazon, AM, located in the lower Rio Negro region, and forest areas in the municipality of Juriti, PA. The captive animals were obtained from the National Centre of Primates (CENP) located in the city of Ananindeua, PA (Table I).

A total of 16 samples of gastric tissue were obtained from captive monkeys that died at different time periods throughout the duration of the study. These animals did not present a history of stomach disease and were fed a balanced diet of fresh fruit, roots, insects and other items in accordance with the natural diet for each species. Some of the samples were obtained from the CENP collection (2004-2005), while others came from autopsies conducted during the study period (2005-2007). The organs from 24 free-living wild monkeys were obtained through donations from other projects in different geographic areas of the Amazon.

Histopathological diagnosis and detection of the genus Helicobacter - All of the specimens analysed were obtained from the antral region of the stomach. The gastric tissue from the primate species was collected in buffered formalin and processed in paraffin. Subsequently, 4-5 µm tissue slices were mounted onto silanised slides for use in histopathological, modified Gram staining (Hucker 1921) and immunohistochemical analyses.

Slides containing the gastric tissue from each animal were stained using the haematoxylin-eosin method for the histopathological diagnosis of gastric tissue and modified Gram staining (Hucker 1921) was used to detect *Helicobacter*-type HLO. These analyses were performed using conventional optical microscopy. The bacteria were characterised by their bacillary (curved and spiralled) and coccoid characteristics.

Immunohistochemistry for detecting H. pylori - The tissue were deparaffinised in xylene, rehydrated in methanol dilutions and washed with phosphate buffer (pH 7.6) followed by antigenic exposure in a citrate buffer (pH 6.0) heated for 15 min in a microwave. The tissues were pre-incubated in blocking buffer [phosphate buffered saline (PBS) and bovine albumin at a 1:20 dilution] for 10 min and subsequently incubated with anti-*H. pylori* antibody (polyclonal rabbit anti-*H. pylori*; DAKO, Denmark B0471) for 1 h at a dilution of 1:100 in blocking buffer (Haqqani 2001). After the primary antibody incubation, the slides were again washed and blocked in blocking buffer. The secondary antibody (polyclonal swine anti-rabbit AP; DAKO D0306) was added at a dilution of 1:80 and the slides were incubated for 1 h. After a final wash with PBS, the staining was visualised with HistoMark RED activating solution (KLP Laboratories, Maryland USA; 556900). The gastric tissue slices were additionally stained with haematoxylin. As a positive control, a sample of human *H. pylori* gastric tissue was used. For the negative controls, the primary antibody was replaced with blocking buffer.

Immunohistochemistry for detecting ABH antigen expression - To determine whether the A, B and H antigens were expressed in the primate organs, a modified

TABLE I

Primate species and origin analysed in this study

Specie	n	Origin (identification of the animal)
Alouatta nigerrima	1	PARNA (An)
Aotus infulatus	2	CENP (Ai_1, Ai_2)
Cacajao melanocephalus	2	RDSA (Cm_1, Cm_2)
Callicebus hoffmanni	2	PARNA (Ch_1-Ch_2)
Callithrix jacchus	5	CENP (Cj_1-Cj_5)
Cebus albifrons	2	PARNA (Cal_1, Cal_2)
Cebus apella	9	CENP (Cap_1-Cap_4); JTR (Cap_5); PARNA (Cap_6-Cap_9)
Mico humeralifer	1	PARNA (Mh)
Pithecia irrorata	2	PARNA (Pi_1, Pi_2)
Saguinus fusicolis	1	CENP (Sf)
Saguinus inustus	3	RDSA (Si_1-Si_3)
Saimiri sciureus	8	CENP (Ss_1 a Ss_4); JTR (Ss_5-Ss_8)
Saimiri vanzolinii	2	RDSM (Sv_1, Sv_2)
Total	40	-

CENP: National Centre of Primates; JTR: municipality of Juriti, state of Pará; PARNA: Amazon National Park; RDSA: Amanã Sustainable Development Reserve; RDSM: Mamirauá Sustainable Development Reserve.

indirect immunoperoxidase technique was used (Pedal et al. 1989, Martins et al. 2006). Antibodies produced commercially for the detection of human antigens were employed. To detect the H antigen, *Ulex europaeus* lectin (Sigma, Switzerland - UEA I) was utilised. The tissue slices were processed, mounted onto histological slides, deparaffinised in xylene and then treated with methanol containing 0.3% H_2O_2. Subsequently, the sections were washed in phosphate buffer (pH 7.6) and blocked in blocking solution (PBS and bovine albumin), followed by a 1-h incubation with anti-A and anti-B monoclonal antibodies (Fresenius Hemocare Brasil) at a 1:10 dilution and peroxidase-conjugated *U. europaeus* lectin (Sigma) diluted 1:50 in blocking buffer at room temperature. All of the slides were washed with phosphate buffer and the slides that were incubated with anti-A and anti-B antibodies were treated with blocking solution followed by a second incubation with peroxidase-conjugated anti-mouse IgM (μ-chain specific - Sigma) for 1 h. After washing in phosphate buffer, the slides were developed in Tris buffer containing diaminobenzidine and hydrogen peroxide. The slides were subsequently stained with haematoxylin, dehydrated and mounted with Entellam (Sigma).

Because all primates are secretors of ABH substances (Apoil et al. 2000), the characterisation of the blood groups was based on the expression of the individual antigens in the stomach epithelium and mucus. This classification method was previously established in another study (Ito et al. 1990) because saliva or other secretions from the animal were not available.

Non-parametric statistical tests (i.e., G-test, Kappa, Fisher tests, among others) were conducted to detect differences between the variations investigated using the computing program BioEstat 5.0 (Ayres et al. 2007). Statistical significance was accepted at the 95% confidence internal level (p value < 0.05).

Ethics - The protocol for the collection and sampling of wild and captive animals was approved by the Federal Environmental Agency (IBAMA 086/2004, 0013/2004 and 001/2008) and the Ethics in Research Committee at the Evandro Chagas Institute (069-2005) Belém, PA, Brazil.

RESULTS

Detection of Helicobacter-type HLO using modified Gram staining - Of the 40 samples of monkeys analysed in this study using the modified Gram staining method, 19 were considered positive for the presence of HLO, eight being from the captive animals and 11 from free-living monkeys.

Among the positive captive primates, three samples were from the species *Cebus apella*, one from *Saimiri sciureus*, three from *C. jacchus* and one from *Aotus infulatus*.

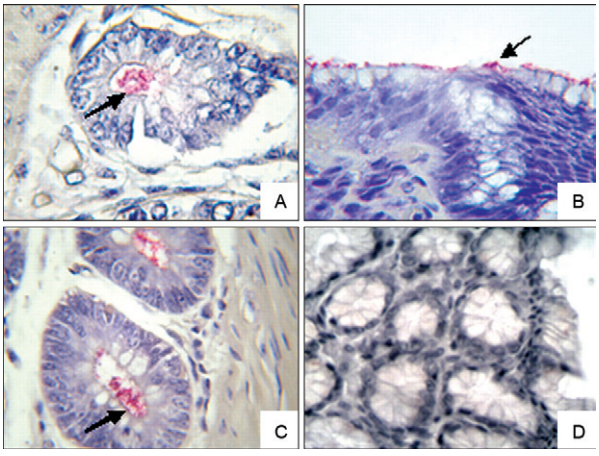
In the free-living group of monkeys, three samples were from *S. sciureus*, two from *C. apella*, one from *Cacajao melanocephalus*, one from *Saguinus inustus*, one from *Saimiri vanzolinii*, one from *Mico humeralifer*, one from *Pithecia irrorata* and one from *Callicebus hoffmanni*.

The binomial test revealed that the proportion of animals that were Gram-positive for HLO did not differ between the free-living and captive animals ($p_{bi} = 0.7960$).

Immunohistochemical detection of H. pylori - When the samples were tested using immunohistochemistry for the specific detection of *H. pylori*, 12 positive samples were found; five samples were from the CENP and seven were from the wild animals group (Figure).

The five CENP animals that were positive for *H. pylori* belonged to the species *A. infulatus*, *C. apella*, *C. jacchus*, *S. sciureus* and *Saguinus fusicolis*; the last two species were negative for *H. pylori* in the modified Gram staining analysis. The wild primates were from the species *S. vanzolinii*, *C. melanocephalus*, *S. inustus*, *C. apella*, *M. humeralifer*, *P. irrorata* and *C. hoffmanni*.

A comparison of the different diagnostic methods used in this study (Table II) indicated that there was significant consistency between the modified Gram staining and immunohistochemical techniques in detecting the *Helicobacter* sp. Notably, the immunohistochemical analysis detects specific epitopes of the bacteria, while the modified Gram staining technique only detects HLO. These analyses were performed using bacteria that were characterised according to their bacillary (curved and spiral) and coccoid characteristics.



A, B: photomicrograph immunohistochemistry stain (arrows) to *Helicobacter pylori* (1,000X); C: positive control reaction (1,000X); D: negative control reaction (400X).

TABLE II

Methods for detecting *Helicobacter* sp. in samples of primates investigated

Test	IHQ positive	IHQ negative	Total
Gram-positive	10	9	19
Gram-negative	2	19	21
Total	12	28	40

MacNemar test by exact method: p (B/C) = 0.0654; IHQ: immunohistochemistry.

The histopathological diagnosis using haematoxylin-eosin staining detected the presence of gastric inflammation in eight captive and 13 wild animals. The results from the statistical tests (Fisher exact) showed that the occurrence of gastritis among free-living and captive animals was not significantly different ($p > 0.05$). Therefore, no significant association between gastritis and the presence of HLO infection was verified through either modified Gram staining and/or immunohistochemical methods (Table III).

All of the animals were characterised in terms of their human-type ABO blood group phenotypes (Table IV). No significant association was found between the phenotypes and the presence of bacterial infection (G-test = 2.8924; $p = 0.4085$).

Expression of the ABH antigens in the gastric mucosa - In this study, the HLO were observed in the regions of the epithelium on the surface of the gastric mucosa, the surface mucus and in the lumen of the gastric glands. Additionally, when normal and inflamed areas of the gastric mucosa from the same individual were compared, changes in the expression pattern of ABH antigens in areas of inflamed tissue were observed as a loss and/or reduction of the staining intensity (data for the ABH antigenic activity not shown).

DISCUSSION

The results of this study are consistent with previous results showing that *Helicobacter* can naturally colonise different primate species (Stadtländer et al. 1998, Solnick et al. 1999, 2003). Previous studies have established infections in New World primates of the genera *Callithrix* and *Saguinus* by new species of the *Helicobacter* genus (Saunders et al. 1999). Recently, Won et al. (2007) described a new species in *C. jacchus*, *H. callitrichis*, which demonstrated that many species of *Helicobacter* are capable of colonising the gastric mucosa of New World primates (Reindel et al. 1999, Mello et al. 2005).

Various methods of histopathological staining are employed for detecting *H. pylori* in humans, such as the silver (Warthin-Starry) and Giemsa stains. In this study, we used the modified Gram staining method because it is widely utilised (Araujo et al. 1993, Pereira et al. 2001, Assumpção et al. 2010) for the detection of bacteria in humans and has also been employed in studies with monkeys (Mackie & O'Rourke 2003).

Indirect immunohistochemistry revealed the presence of specific epitopes for *H. pylori* in 30% of the apes (12/40), while modified Gram staining detected bacteria with the same bacillary morphology as *Helicobacter* in almost half of the animal specimens tested (19/40); thus, new species of this genus might be present in New World primates.

Confirmation of the presence of HLO should not be restricted to a single diagnostic technique and therefore, immunohistochemistry was performed using an antibody specific for *H. pylori*. However, other studies (Loffeld et al. 1991, Jonkers et al. 1997) conducted to test the specificity of this antibody have shown cross-reactivity with the *Campylobacter* sp., a bacterium that does not colonise the gastric environment. Moreover, Reindel et al. (1999) have shown that this antibody cross-reacts with the species *Helicobacter helmanii*, a bacterium normally found in the stomachs of dogs, cats and pigs, which has also been detected in primates (Fox & Lee 1997). Thus, in this study, we cannot exclude the possibility of the presence of this and other species of *Helicobacter* in some of the cases investigated that were detected by the anti-*H. pylori* antibody in the immunohistochemical analysis. Furthermore, other common epitopes that might be shared between *H. pylori* and new species of *Helicobacter*, although not yet identified systematically, could also be recognised by the antibody (Mello et al. 2005).

It was not possible to establish any relationship between inflammation and *Helicobacter* sp. infection because some of the monkeys that were negative by modified

TABLE III

Detection of inflammation of the gastric mucosa and of the *Helicobacter* sp. bacteria by means of Gram-modified and immunohistochemistry methods in the primate study groups

		Detection methods				
Animals	Gastritis	Gram+/ IHQ+	Gram+/ IHQ-	Gram-/ IHQ+	Gram-/ IHQ-	Total
VL	Present	3	3	0	7	13
	Absent	4	1	0	6	11
CAT	Present	2	2	2	2	8
	Absent	1	3	0	4	8
Total	-	10	9	2	19	40

CAT: captivity; IHQ: immunohistochemistry; VL: wild-living.

TABLE IV

Phenotypes of the ABO blood groups in the species studied

	ABO phenotypes			
Species	A	B	AB	O
Callithrix jacchus, *Mico humeralifer*, *Pithecia irrorata*, *Saguinus inustus*, *Saguinus fusicolis*	12	-	-	-
Alouatta nigerrima, *Aotus infulatus*, *Saimiri vanzolinii*	-	5	-	-
Cacajao melanocephalus	1	-	1	-
Callicebus hoffmanni	1	-	1	-
Cebus albifrons	1	1	-	-
Cebus apella	3	3	1	2
Saimiri sciureus	3	1	4	-
Total	21	10	7	2

Gram staining and/or immunohistochemical analysis had gastric inflammation. Therefore, it is rational to conclude that the helicobacteriosis in the gastric mucosa of these primates is not a primary factor for inducing an inflammatory response. Furthermore, in the stomach lesions of the monkey species investigated, an infiltrate of mononuclear cells and the presence of lymphoid follicles was not common. Thus, the observed inflammation might be associated with other etiological factors (Khanolkar-Gaitonde et al. 2000) because gastritis origins are of a multifactorial nature. For example, both wild and captive primates suffer from stress induced by the maintenance of the colony's social hierarchy, a known factor in the onset of gastritis (Tamashiro et al. 2005).

There was no association detected between the genetic markers for the ABO blood group system phenotypes and bacterial infection or the inflammatory process. However, previous studies have demonstrated that various *H. pylori* strains might be associated with specific fucosylated ABH antigens in the gastric tissue of both human and non-human primates (Mahdavi et al. 2002, Aspholm-Hurtig et al. 2004, Lindén et al. 2004, Styer et al. 2010). However, the expression of these ABH antigens was reduced in the gastric mucosa of some of the animals in this study, which suggests that the inflammation and other associated infections might affect the tissue densities of specific ABH antigens, which *H. pylori* uses as receptors and therefore influence susceptibility to this infection. Notably, the small sampling number reduced the efficacy of the test and the relationship between the susceptibility of the host and the pathogen was statistically insignificant.

Non-human primates, especially those from the Old World, have been used as models for the study of various infectious diseases in humans because they are also susceptible to many pathogens. This susceptibility is associated with the evolutionary relationship within the primate order. The results of this study seem to indicate that various species of New World monkeys have similar risks for natural infection by the *Helicobacter* sp. Thus, this study provides a comparative analysis of the *Helicobacter* infection between human and non-human primates that results in the identification of new species in these hosts and increases our understanding of the pathogenic mechanisms of helicobacteriosis.

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