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.
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 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

<table>
<thead>
<tr>
<th>Specie</th>
<th>Origin (identification of the animal)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alouatta niggerrima</td>
<td>PARNA (An)</td>
<td>1</td>
</tr>
<tr>
<td>Aotus infilatus</td>
<td>CENP (Ai, Ai)</td>
<td>2</td>
</tr>
<tr>
<td>Cacajao melanocephalus</td>
<td>RDSA (Cm, Cm)</td>
<td>2</td>
</tr>
<tr>
<td>Callitcebus hoffmannii</td>
<td>PARNA (Kh, Kh)</td>
<td>2</td>
</tr>
<tr>
<td>Callithrix jacchus</td>
<td>CENP (Cj, Cj)</td>
<td>5</td>
</tr>
<tr>
<td>Cebus albifrons</td>
<td>PARNA (Ca, Ca)</td>
<td>2</td>
</tr>
<tr>
<td>Cebus apella</td>
<td>CENP (Ca, Ca); JTR (Cap); PARNA (Cap)</td>
<td>9</td>
</tr>
<tr>
<td>Mico humalifer</td>
<td>PARNA (Mh)</td>
<td>1</td>
</tr>
<tr>
<td>Pithecia irrorata</td>
<td>PARNA (Pi, Pi)</td>
<td>2</td>
</tr>
<tr>
<td>Saguinus fuscicolor</td>
<td>CENP (Sf)</td>
<td>1</td>
</tr>
<tr>
<td>Saguinus inustus</td>
<td>RDSA (Si, Si)</td>
<td>3</td>
</tr>
<tr>
<td>Saimiri sciureus</td>
<td>CENP (Ss, Ss); JTR (Ss, Ss)</td>
<td>8</td>
</tr>
<tr>
<td>Saimiri vanzolinii</td>
<td>RDSA (Sv, Sv)</td>
<td>2</td>
</tr>
</tbody>
</table>

Total 40 -

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 melanocepalus, one from Saginus inustus, one from Saimiri vanzolini, 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 = 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 Saginus fusicolis; the last two species were negative for H. pylori in the modified Gram staining analysis. The wild primates were from the species S. vanzolini, C. melanocepalus, 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.

<p>| Table II |
| Methods for detecting Helicobacter sp. in samples of primates investigated |</p>
<table>
<thead>
<tr>
<th>Test</th>
<th>IHQ positive</th>
<th>IHQ negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram-positive</td>
<td>10</td>
<td>9</td>
<td>19</td>
</tr>
<tr>
<td>Gram-negative</td>
<td>2</td>
<td>19</td>
<td>21</td>
</tr>
<tr>
<td>Total</td>
<td>12</td>
<td>28</td>
<td>40</td>
</tr>
</tbody>
</table>

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 Saimirius 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 (Löffeld 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

<table>
<thead>
<tr>
<th>Animals</th>
<th>Gastritis</th>
<th>Detection methods</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Gram+/IHQ+</td>
</tr>
<tr>
<td>VL</td>
<td>Present</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Absent</td>
<td>4</td>
</tr>
<tr>
<td>CAT</td>
<td>Present</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Absent</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>

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

### TABLE IV

Phenotypes of the ABO blood groups in the species studied

<table>
<thead>
<tr>
<th>Species</th>
<th>ABO phenotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Callithrix jacchus, Mico humeralifer, Pithecia irrorata, Saguinus inustus, Saimiri vanzolinii</td>
<td>12 - - -</td>
</tr>
<tr>
<td>Alouatta nigerrima, Aotus infalutus, Saimiri sciureus</td>
<td>- 5 - -</td>
</tr>
<tr>
<td>Cacajao melanocephalus</td>
<td>1 1 1</td>
</tr>
<tr>
<td>Callicebus hoffmanni</td>
<td>1 1 1</td>
</tr>
<tr>
<td>Cebus albifrons</td>
<td>3 3 1 2</td>
</tr>
<tr>
<td>Cebus apella</td>
<td>3 1 4 -</td>
</tr>
<tr>
<td>Saimiri sciureus</td>
<td>1 4 -</td>
</tr>
<tr>
<td>Total</td>
<td>21 10 7 2</td>
</tr>
</tbody>
</table>

H. callitrichis, a bacterium that does not colonise humans and has also been employed in studies with monkeys (Mackie & O’Rourke 2003).

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