A comparison of Helicobacter pylori and non - Helicobacter pylori Helicobacter spp. Binding to Canine Gastric Mucosa with Defined Gastric Glycophenotype

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ABSTRACT

Background

The gastric mucosa of dogs is often colonized by non - Helicobacter pylori helicobacters (NHPH), while H. pylori is the predominant gastric Helicobacter species in humans. The colonization of the human gastric mucosa by H. pylori is highly dependent on the recognition of host glycan receptors. Our goal was to define the canine gastric mucosa glycophenotype and to evaluate the capacity of different gastric Helicobacter species to adhere to the canine gastric mucosa.

Materials and Methods

The glycosylation profile in body and antral compartments of the canine gastric mucosa, with focus on the expression of histo - blood group antigens was evaluated. The in vitro binding capacity of FITC - labeled H. pylori and NHPH to the canine gastric mucosa was assessed in cases representative of the canine glycosylation pattern.

Results

The canine gastric mucosa lacks expression of type 1 Lewis antigens and presents a broad expression of type 2 structures and A antigen, both in the surface and glandular epithelium. Regarding the canine antral mucosa, H. heilmannii s.s. presented the highest adhesion score whereas in the body region the SabA - positive H. pylori strain was the strain that adhered more.

Conclusions

The canine gastric mucosa showed a glycosylation profile different from the human gastric mucosa suggesting that alternative glycan receptors may be involved in Helicobacter spp. binding. Helicobacter pylori and NHPH strains differ in their ability to adhere to canine gastric mucosa. Among the NHPH, H. heilmannii s.s. presented the highest adhesion capacity in agreement with its reported colonization of the canine stomach.

INTRODUCTION

The gastric mucosa of dogs is often colonized by helicobacters different from Helicobacter pylori (H. pylori). These non - H. pylori helicobacters (NHPH) are present in 67-86% of clinically healthy dogs, in 61-100% of animals presenting chronic vomiting 1-5 and in about 100% of laboratory dogs and dogs from local shelters 6-8. The predominant gastric Helicobacter species in dogs are H. felis, H. bizzozeronii, and H. heilmannii sensu stricto (s.s.), while H. salomonis is less often detected and the prevalence of H. cynogastricus and H. baculiformis has not yet been studied 9-12. Mixed infections with different species can also occur 13. The pathogenic significance of gastric NHPH in dogs is poorly understood and remains controversial; therefore veterinarians are facing the dilemma of either treating or ignoring spiral organisms observed in canine gastric biopsies. However, these NHPH are of zoonotic significance and have been associated with gastritis, peptic ulcers and mucosa associated lymphoid tissue lymphomas in human patients 11. Dogs may constitute a source of infection for their owners, although the prevalence of these canine - and feline - associated helicobacters in humans is much lower than that of H. pylori. On the other hand, H. pylori has occasionally been identified in the canine stomach 14, 15. Gastric Helicobacter species have a host species preference, but may occasionally cross this host species barrier. The molecular mechanisms underlying this partial host - adaptation are not known, but may be related to differences in glycosylation profile of the gastric mucosa.

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The gastrointestinal tract glycosylation profile determines the colonization capability of various infectious agents. Bacterial binding occurs through recognition of specific glycan receptors expressed by the host epithelial cells 16-18. The biosynthesis of glycan chains is controlled by enzymatic activity of several glycosyltransferases, which are expressed in a cell, tissue and species - specific manner (for review see 19).

The expression of the terminal Lewis glycan structures that are recognized as receptors for H. pylori in human gastric tissue depends on the enzymatic activity of specific fucosyltransferases and sialyltransferases 20 and are schematically represented in Fig. 1A, B. Two backbone structures are the precursors for the Lewis antigens biosynthesis: the type 1 Gal β 1 - 3GlcNAc, and the type 2 Gal β 1 - 4GlcNAc chains. Addition of a fucose to terminal galactose on type 1 structures leads to H - type 1 structure, which can be further modified with a fucose on the GlcNAc residue resulting in Lewis b (Leb). Alternatively, the type 1 backbone may be fucosylated on the GlcNAc residue leading to Lewis a (Lea) structure. Furthermore, the action of a sialyltransferase toward type 1 chains can lead to the biosynthesis of the sialyl - Lea (sLea) antigen. The biosynthesis of type 2 based Lewis antigens depends on the addition of the same glycan units but with different linkages, originating the isomers Lewis x (Lex), Lewis y (Ley) and sialyl - Lex (sLex) antigens (Fig. 1B) (as reviewed by 20).

The H. pylori blood group antigen - binding adhesin (BabA) recognizes both H - type 1 and Leb fucosylated antigens 16 expressed on the surface of the gastric mucosa of secretor individuals 21, 22. The secretor status is determined by the activity of the fucosyltransferase 2 enzyme (FUT2) 23 and the capacity to synthesize H - type 1 and Leb antigens in body secretions. The secretor and Lewis status in humans are associated with the adhesion and infection of H. pylori strains expressing the BabA adhesin 21, 24, 25. In addition, a second H. pylori adhesin, the sialic acid - binding adhesin (SabA) has been described, which recognizes the sialylated antigens sLea and sLex 17. These sialylated structures are absent in normal human gastric mucosa but are induced upon H. pylori infection and gastric mucosa inflammation 17, 26, 27.

The canine secretory alloantigen alloantibody system (CSA) is closely related to the human ABH - Le system being equivalent to the major human ABO exocrine glycophenotype. The CSA was first described in 1966 by Zweibaum and colleagues 28. The genetic polymorphisms of the CSA were later analysed 29 and the structure of canine polymorphic antigens was further characterized in four phenotypes: A, X, Y, and AY 30 (Fig. 1C). However, limited information is available about the glycosylation profile of the canine gastric mucosa.

Here we describe, for the first time, the glycophenotype observed in the canine gastric mucosa, with focus on the expression of Lewis glycan antigens. Furthermore, the binding capacity of H. pylori and NHPH to canine gastric mucosa was evaluated. This study provides valuable information about the canine gastric glycosylation profile and about the Helicobacter capacity to adhere to the canine gastric mucosa, contributing to the understanding of the host colonization spectrum of the different Helicobacter species.

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MATERIALS AD METHODS

Tissue Samples and Histology

Gastric samples were obtained during necropsy procedures from dogs that died from non - infectious causes (eight representative sections of the body and six of the antrum of the stomach).

Tissues were fixed in 10% buffered formalin and paraffin - embedded. Serial sections 3 μm thick were made, one being stained with haematoxylin and eosin (HE) for histopathology and the others were used for the histochemical and immunohistochemical studies.

Sections stained with HE were examined by three pathologists and were considered as normal gastric tissues, according to the criteria proposed by Prachasilpchain et al. 31. Additionally, the presence of Helicobacter spp. was excluded based on the negative results of modified Giemsa stain and of anti - H. pylori immunohistochemistry, using a polyclonal antibody (RBK012; Zytomed, Berlin, Germany), which shows immunoreactivity with a wide range of bacteria belonging to the Helicobacter genus.

Immunohistochemistry

For the immunohistochemical study, sections were deparaffinized, hydrated and antigen retrieval was performed in a pressure cooker in 10 mmol/L sodium citrate buffer, pH 6.0, for 2 minutes. Slides were cooled for 10 minutes at room temperature and rinsed twice in triphosphate buffered saline (TBS) for 5 minutes. The Novolink™ Max - Polymer detection system (Novocastra, Newcastle, UK) was used for visualization, according to the manufacturer′ s instructions. After blocking endogenous peroxidase with 3% hydrogen peroxide in methanol for 10 minutes, sections were incubated, overnight at 4 °C, with the monoclonal antibodies specific for the carbohydrate antigens (Table 1). Sections were rinsed with TBS between each step of the procedure. Color was developed for up to 7 minutes at room temperature with 3,3′ - diamino - benzidine (DAB; Sigma, St. Louis, MO, USA) and sections were then lightly counterstained with haematoxylin, dehydrated, and mounted.

Sections of human gastric mucosa with intestinal metaplasia were obtained from the pathology department archive of Hospital Santo Antonio – Porto and were used as positive controls. Negative controls were performed by replacing the primary antibody with an antibody of the same immunoglobulin isotype.

Lectin Staining

The biotinylated lectin Dolichus biflorus agglutinin (DBA), for detection of terminal alpha linked GalNAc, and the avidin - biotin - peroxidase complex (Vectastain Elite ABC kit) solution were purchased from Vector Laboratories (Burlingame, CA, USA). After deparaffination and rehydration, endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 10 minutes at room temperature. After washing twice with phosphate buffered saline (PBS) for 5 minutes, sections were incubated with 10% bovine serum albumin (BSA) in PBS for 30 minutes at room temperature and then in the biotinylated lectin solution (1 : 100 in PBS) for 1 hour, at room temperature. The slides were subsequently washed in PBS and immersed in ABC solution for 30

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minutes at room temperature, according to manufacturer′ s recommendations. After washing twice with PBS for 5 minutes, slides were immersed in a freshly prepared solution of DAB containing 0.02% hydrogen peroxide for 7 minutes at room temperature to visualize the lectin binding sites, counterstained with haematoxylin, dehydrated and mounted. Sections of canine normal intestine were used as positive controls. Negative controls were performed by replacing the lectin by PBS.

Evaluation of Gastric Tissue Glycosylation Profile

A microscopic evaluation was performed by analyzing the entire section of the gastric tissue. Positive immunoreactivity was recorded as a distinct brown labeling of cytoplasm and/or membrane of epithelial cells. Extracellular staining of the superficial mucus was excluded from our evaluation. The immunostaining was scored according to the intensity (0, absent; +, weakly positive; + +, moderately positive; or + + +, intense stain) and distribution (sparse, focal, multifocal, and generalized).

Bacterial Strains

The H. pylori strains 17875/Leb and 17875babA1::kan babA2::cam (17875babA1A2) were obtained from the Department of Medical Biochemistry and Biophysics, Umeå University, Sweden 17. These model strains were selected based on their capacity to adhere to human gastric mucosa tissue sections. The 17875/Leb strain is a spontaneous mutant which expresses both BabA and SabA adhesins, however, does not show sialylated dependent binding. The 17875babA1A2 is a BabA adhesin mutant strain which expresses a functional SabA adhesin 17.

Helicobacter pylori were grown in Pylori agar (BioMérieux, Marcy l'Étoile, France) at 37 ° C under microaerobic conditions (85% N2, 10% CO2, 5% O2; 37 ° C). For strain babA1A2 - mutant, culture media included also 20 mg/L Chloramphenicol (Sigma) and 25 mg/L Kanamycin (Sigma).

The NHPH strains were provided by the Department of Pathology, Bacteriology and Avian Diseases, Faculty of Veterinary Medicine, Ghent University. The H. felis CS1, H. salomonis R1053 and H. bizzozeronii R1051 strains were cultured biphasically on Brain Haert Infusion agar plates (BHI; Oxoid, Basingstoke, UK) supplemented with 10% horse blood, 20 μg/mL amphotericin B (Fungizone; Bristol - Myers Squibb, Eppernon, France), Vitox supplement (Oxoid), and Campylobacter selective supplement (Skirrow, containing 10 μg/mL vancomycin, 5 μg/mL trimethoprim lactate, and 2.5 U/mL; Oxoid). The H. heilmannii strain ASB1.4 was cultivated biphasically on Brucella agar plates (Oxoid) supplemented with 20% (v/v) fetal calf serum (HyClone, Logan, UT), 5 mg/L amphothericin, Skirrow and Vitox supplements (Oxoid), and 0.05% HCl (pH 5). All strains were incubated under microaerobic condition.

In vitro Binding Assay of Helicobacter pylori and NHPH to Canine Gastric Mucosa

For the in vitro Helicobacter binding assay we selected three representative cases (n = 3) of the general canine glycosylation pattern, belonging to three different dogs, in which body and antrum samples were available.

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The different Helicobacter strains were labeled with fluorescein isothiocyanate (FITC) and concentration adjusted as previously described 32. Adhesion assays were performed as previously described 22, 32 with the following modifications. Briefly, paraffin embedded canine gastric sections were deparaffinized and rehydrated followed by overnight incubation at 4 °C and 1 hour at room temperature, with blocking buffer: 1% BSA in PBS containing 0.05% Tween 20. BSA was previously submitted to Periodate oxidation to destroy competitive carbohydrate receptors for Helicobacter binding 33. The FITC - labeled bacterial suspension was diluted 5 - fold in blocking buffer and 100 μ L was used to incubate each section for 2 hours at room temperature. Slides were subsequently washed five times with PBS containing 0.05–0.1% Tween 20 and stained with DAPI. Evaluation was estimated by the number of adhered bacteria under 100× magnification, and analyzing at least five different fields per case and quantified using the ImageJ software (NIH, Bethesda, MD, USA).

Statistical Analysis

The Student's t - test was used to compare the differences between the mean adhesion values of both stomach regions for the same NHPH strain. The significance of differences between mean values of each Helicobacter strain was assessed using a one - way ANOVA (GraphPad Prism, GraphPad Software, Inc., La Jolla, CA, USA). p values < 0.05 were considered as statistically significant.

RESULTS

Expression of Type 1 Lewis Antigens in Dog Gastric Mucosa

There was no Lea antigen expression in the body region of the stomach from seven of the eight canine cases studied (Fig. 2A, Table 2). Only in one case, a very weak and sparse Lea expression was detected in few superficial foveolar epithelial cells. In the antral region of the stomach, there was no Lea antigen expression in all six cases studied (Fig. 2B, Table 2). Lewisb and sLea expression were absent in both the body and antrum of the stomach (Fig. 2D,E,G,H, Table 2).

Expression of Type 2 Lewis Antigens in Dog Gastric Mucosa

In the body of the stomach, expression of Lex was seen in five of the eight canine cases (Table 2). The staining intensity varied from moderate to strong, always limited to the superficial foveolar epithelium (Fig. 2J). In all positive cases, the staining was multifocal and restricted to the cytoplasm. In the antrum of the stomach, Lex expression was found in all cases. The staining intensity varied from weak to strong and was limited to the superficial foveolar epithelium. The pattern of staining was multifocal in four cases and generalized in the other two cases (Fig. 2K, Table 2).

All cases evaluated presented Ley in the body and antrum. In the body, the level of immunostaining was weak in one case and intense in seven cases. The staining included superficial foveolar epithelium and parietal cells (Fig. 2M). The distribution of the staining was multifocal in four cases and generalized in the other four. The subcellular staining was mainly localized in the cytoplasm of the cells. In the antrum, the level of Ley immunostaining varied from moderate to intense, presenting stronger labeling in the cells of the foveolar epithelium and gastric pits. The pattern of staining was generalized in all cases (Fig. 2N, Table 2).

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In the body of the stomach, sLex expression was noted in four cases. The level of immunostaining varied from weak to strong. The staining included superficial foveolar epithelium (Fig. 2P). The staining distribution was multifocal in all cases studied. The subcellular staining was mainly localized in the cytoplasm of the cells. In the antrum of the stomach, a sparse and moderate expression of sLex was observed in only one case. The staining was limited to the cytoplasm of the foveloar superficial epithelium and cells of the pyloric glands (Fig. 2Q, Table 2).

Expression of Terminal α - GalNAc in Dog Gastric Mucosa

In the body of the stomach, expression of terminal α - GalNAc was found in all canine cases, as detected by DBA lectin labeling. The staining was moderate in three cases and intense in the remaining five cases. The staining included superficial foveolar epithelium and parietal cells (Fig. 2S). The staining pattern was generalized in all cases. The subcellular staining was localized in the cytoplasm of the cells. In the antrum of the stomach, generalized expression of terminal α - GalNAc was detected in all cases. The level of staining was moderate in four cases (Fig. 2T) and intense in the other two. The staining included the foveolar epithelium, gastric pits and pyloric glands. Generally, the superficial pyloric glands showed an intense staining higher than that recorded in the deeper glands; only in one case the staining was identical in both areas. The subcellular staining was localized in the cytoplasm of the cells. (Table 2).

In vitro Binding Assay of Helicobacter pylori and NHPH to Canine Gastric Mucosa

The FITC labeled Helicobacter strains were verified for size and morphology in accordance with literature: H. pylori strains measured about 2.5–5 µm in length and 0.5–1 µm in width (Fig. 3A,B); The NHPH strains were larger and apparently more tightly coiled spiral - shaped bacteria: ASB1.4 was, approximately 3-6.5 μ m long and 0.6-0.7 μ m wide (Fig. 3C); CS1 measured about 5-7.5 μ m in length and 0.4 in width (Fig. 3D) while R1051 ranged from 5 to 10 μ m in length and 0.3 in width (Fig. 3E). R1053 was less spiral, measuring 5–7 µm in length and 0.8–1.2 µm wide (Fig. 3F) (as reviewed by 34). The length was variable depending on the state of contraction.

Regarding tissue sections from the body of canine stomach, we observed that the H. pylori strain 17875babA1A2 (SabA+) had the highest adhesion. In contrast, the H. pylori strain 17875/Leb (BabA+) showed low levels of adhesion. Among the NHPH strains evaluated, ASB1.4 presented the highest adhesion value, followed by CS1 and R1051. The R1053 was the strain which adhered less being possible to observe gastric regions completely devoid of bacterial adhesion (Fig. 4).

In the antral sections, the H. pylori strains 17875babA1A2 and 17875/Leb showed a similar binding behavior, exhibiting very closely means values of adhesion. The quantification revealed that ASB1.4 was the strain that presented highest adhesion values to the canine antrum tissue sections. Additionally, CS1 and R1051 strains adhered similarly to canine antral mucosa, whereas R1053 adhered less (Fig. 5).

The ASB1.4 adhesion value quantified in the antral mucosa was significantly higher from that obtained in the body region of the canine stomach ($p < .05$). No significant differences were found in CS1, R1051, and R1053 bacterial adhesion, concerning both canine stomach compartments.

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DISCUSSION

In the present study, we have characterized the expression of histo - blood group antigens in the normal canine gastric mucosa to define the gastric glycosylation profile of dogs (Table 2). The expression pattern of type 1 and type 2 Lewis antigens in the canine gastric mucosa revealed a different distribution when compared with the human gastric mucosa. Normal canine mucosa showed minor expression of type 1 Lewis antigens, with absence of Leb and sLea expression and only a sparse expression of Lea in the body of one of the animals tested. In the human normal gastric mucosa, and depending on the secretor status of the individual, Lea and Leb are highly expressed in the superficial foveolar epithelium 23, 35, where MUC5AC mucin is co - expressed 36. The normal canine mucosa demonstrated expression of Lewis type 2 antigens characterized by expression of Lex and Ley, along with a minor expression of sLex. In the canine gastric mucosa this expression was mostly observed in the superficial and foveolar epithelium, with Ley expression extending to the deeper portions of the gastric gland. In the human gastric mucosa, the expression of Lewis type 2 antigens is generally restricted to the deeper part of the gastric glands, where MUC6 mucin is co - expressed 37, 38. Knowing that type 1 fucosylated structures are receptors for H. pylori in human stomach, the different expression patterns of type 1 and 2 Lewis antigens observed may play a role in differences in prevalence of gastric helicobacters in both mammalian species.

The pattern of Lewis antigen expression observed in canine gastric mucosa is in agreement with a previous study which reported a prominent representation of type 2 chains in dog intestinal secretions 39.

According to our results, the A - antigen is also extensively expressed in the normal canine gastric mucosa, representing an important component of the dog gastric mucosa glycophenotype (Table 2). This evidence is in accordance with earlier observations documenting that this antigen is expressed in canine gastrointestinal tissues 30. Interestingly, the pattern of expression of the A - antigen in canine gastric mucosa resembles the reactivity of DBA lectin previously reported in human gastric tissue 40. However, its putative involvement in Helicobacter adhesion has never been addressed.

Lewis antigens biosynthesis is dependent on the cells repertoire of glycosyltransferases, namely fucosyltransferases. The absence of type 1 Lewis antigens in dog gastric mucosa may be explained by the lack of expression of enzymes with α1,4 fucosyltranferase activity. The human enzymes with this activity, FUT3 and FUT5, seem to have originated late in hominid evolution from duplication of an ancestor Lewis gene present in lower mammals 41. Moreover, it has been demonstrated that the acceptor substrate specificity of vertebrate α 1,3/1,4 - fucosyltransferases is determined by a single amino acid in the hypervariable stem domain 42. Most vertebrate α 1,3 fucosyltransferases present a conserved arginine in this domain while a tryptophan residue is present at the same position on human FUT3 and FUT5. Noteworthy, the dog gene orthologous of the human FUT3 and FUT5 gene presents at this position an Arg residue, compatible with α1,3 fucosyltranferase activity and therefore type 2 Lewis synthesis (Fig. S1).

The host glycan receptors are recognized by Helicobacter outer membrane proteins (OMPs) which mediate several important host - pathogen interactions 18. Recently, it has been shown that NHPH OMPs genes share sequence homology with H. pylori. Interestingly, it was found that H. acinonychis, a NHPH colonizing big cats, express BabA adhesin 43.

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However, among the NHPH strains included in this study, none presented in their genomes orthologues of H. pylori adhesins. H. bizzozeronii and H. mustelae lack homologs of major H. pylori virulence genes BabA/B and SabA while H. suis lacks BabA/B and SabA/B 44-46. The genome of H. heilmannii s.s. showed that this strain encodes several OMPs but lacks the Bab and Sab adhesins 47.

The absence of homologues of the BabA in these NHPH may underlie the low rate of human infection by these organisms and suggests that other adhesins with different receptor specificity may play a role in the colonization of NHPH. Additionally, the lack of Lewis type 1 antigens expression in the canine gastric mucosa might explain why H. pylori rarely colonizes the stomach of dogs.

To determine if the glycosylation profile of canine gastric mucosa was related to the host - adaptation of the different NHPH, we evaluated the binding capacity of some Helicobacter species to canine gastric mucosa.

Our results show that H. heilmannii s.s. adhered most to the canine gastric mucosa. This result highlights the contribution of adhesion in the canine gastric colonization by this species.

In 2012, H. heilmannii was isolated and described as a novel species 34. The name H. heilmannii sensu stricto (s.s.) was proposed to refer to the novel Helicobacter species and the term H. heilmannii sensu lato (s.l.) to refer to the whole group NHPH 12. Since the discovery and description of H. heilmannii s.s. is still relatively recent, the majority of the epidemiological data available does not comprise the identification of this particular species in the stomach of dogs and cats. This impairs the comparison of the adhesion capacity achieved in this experiment with the spontaneous incidence of H. heilmannii s.s. in dogs.

We also found that the H. heilmannii s.s. adhesion levels were higher in the antrum than in the body region of canine stomach ($p < .05$). In agreement with our observations, Joosten et al. 48 recently studied bacterium - host interactions of 9 H. heilmannii s.s. strains in Mongolian gerbils and concluded that, in general, the colonization capacity in the fundus was lower than in the antrum for all strains tested. Additionally, a clear association was observed between the colonization capacity of the H. heilmannii s.s. strains and the gastric inflammation scores in the antrum of the stomach. In dogs, while many studies reported that the fundus and body present higher bacteria density and higher probability to find Helicobacter spp. 8, 49, 50 others found no significant differences between the density of NHPH in the fundus, body and antrum 31, 51-54. The discrepancies in the results regarding the spatial distribution of Helicobacter spp. in canine gastric mucosa can be attributed to the different laboratorial approaches and diagnostic methodologies used by the various research groups.

The other NHPH strains tested showed lower adhesion levels to the canine gastric mucosa. In comparison to H. heilmannii s.s., H. felis has a lower adhesion capacity, followed by H. bizzozeronii and H. salomonis and this trend was observed in the body as well as in the antrum.

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The two H. pylori strains used in this study are model strains frequently used in adhesion studies due to their well - defined binding properties. In both stomach regions, the H. pylori strain 17875/Leb, which is a BabA positive strain, adhered less than H. heilmannii s.s. This can be explained by the fact that dogs do not express Lewis type 1 antigens, including the BabA - ligand Leb. The H. pylori 17875babA1A2, which is a SabA positive strain, adhered considerably in the body region. This result is in accordance with the concomitant observed expression of sLex, the ligand of the SabA adhesin. However, in vivo and probably due to competitive factors between bacteria and/or different immunological responses of the host, H. pylori often fails to colonize the canine gastric mucosa.

Our results support that the differences in gastric mucosa glycosylation profile play a role in the host adaptation of gastric Helicobacter species. The defined canine gastric mucosa glycophenotype indicates that adhesion of NHPH involves different adhesion mechanisms mediated by proteins with alternative receptor specificity. The knowledge of the adhesion abilities of different Helicobacter strains to canine gastric mucosa is of outmost relevance for the understanding of the physiopathology of Helicobacter infection in the dogs.

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FIGURES

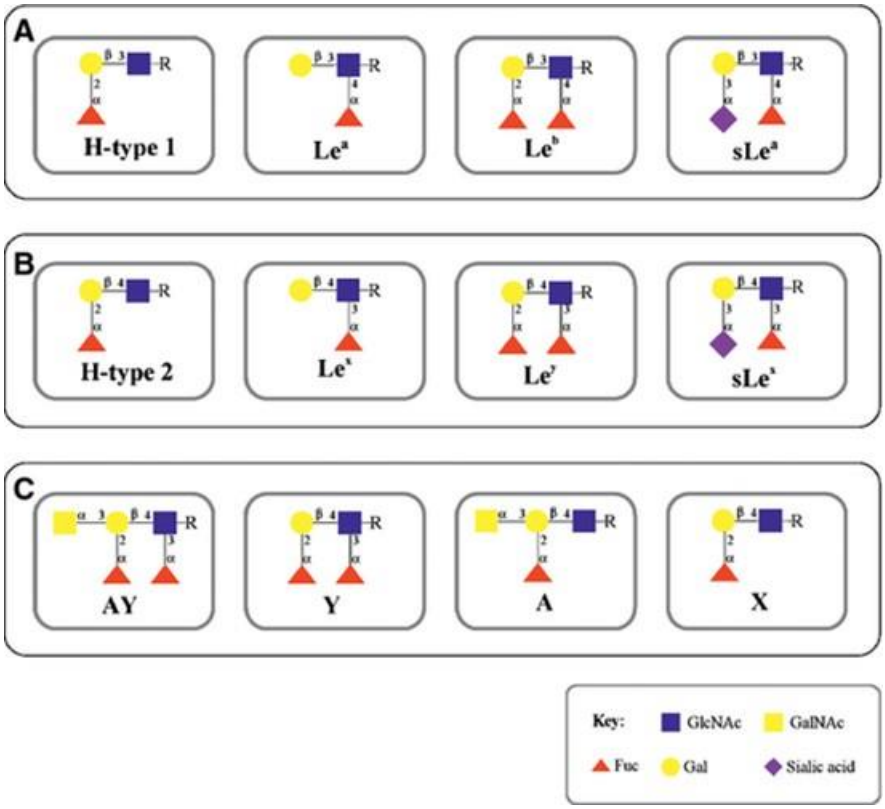

Figure 1. Schematic representation of Lewis antigens. Type 1 chains (panel A) are characterized by the Gal β 1,3 linkage, while type 2 chains (panel B) display a Gal β 1,4 linkage. (Panel A) Addition of a fucose to terminal galactose on type 1 structures leads to H - type 1 structure, which can be further modified with a fucose on the GlcNAc residue resulting in Leb antigen. The type 1 backbone may be just fucosylated on the GlcNAc residue leading to Lea antigen biosynthesis. The action of a sialyltransferase towards the type 1 chain can lead to the biosynthesis of the sLea antigen. (Panel B) the biosynthesis of type 2 based Lewis antigens depends on the addition of the same glycan units but with different linkages, originating the isomers Lex, Ley and sLex antigens. Addition of a fucose to terminal galactose on type 2 structures leads to H - type 2, which can be further modified with a fucose on the GlcNAc residue resulting in Ley antigen. The type 2 backbone may be just fucosylated on the GlcNAc residue leading to Lex antigen biosynthesis. The action of a sialyltransferase toward the type 2 chain can lead to the biosynthesis of the sLex antigen. (Panel C) Schematic representation of canine secretory alloantiagens (CSA) polymorphisms (adapted from Oriol et al. 30).

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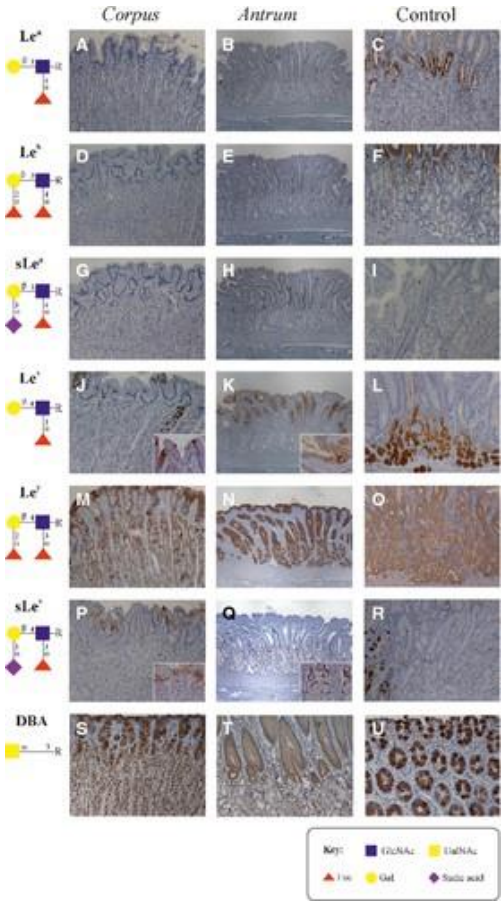

Figure 2. Expression of Lewis antigens and Dolichus biflorus agglutinin (DBA) lectin staining in gastric mucosa: (A) and (B) Normal canine gastric mucosa, IHC Lea, 100× and 40× respectively. There was no Lea antigen expression in both body and antrum. (D) and (E) Normal canine gastric mucosa, IHC Leb, 100× and 40× respectively. There was no Leb antigen expression in both body and antrum. (G) and (H) Normal canine gastric mucosa, IHC sLea, 100× and 40×, respectively. There was no sLea antigen expression in both body and antrum. (J) Normal canine gastric body, IHC Lex, 100×. Multifocal and moderate cytoplasm expression of Lex in the superficial foveolar epithelium. (K) Normal canine gastric antrum, IHC Lex, 40×. Generalized and moderate staining of the superficial foveolar epithelium. (M) Normal canine gastric body, IHC Ley, 100×. Generalized and intense immunostaining of superficial foveolar epithelium andparietal cells. (N) Normal canine gastric antrum, IHC Ley, 40×. Intense and generalized expression of Ley in foveolar epithelium and gastric pits. (P) Normal canine gastric body, IHC sLex, 100×. Multifocal and intense immunostaining of superficial foveolar epithelium. (Q) Normal canine gastric antrum, IHC sLex, 40×. Sparse and weak immunostaining of the foveloar superficial epithelial cells and in cells of the pyloric glands. (C), (F), (I), (L), (O), (R) Expression of Lewis antigens in human gastric mucosa with intestinal metaplasia used as positive controls, 100×. (S) Normal canine gastric body, Dolichus biflorus agglutinin (DBA), 100×. Generalized and intense staining of superficial foveolar epithelium and parietal cells. (T) Normal canine gastric antrum, DBA, 100×. Intense and generalized staining of foveolar epithelium and gastric pits, contrasting with lesser intensity in deeper pyloric glands. (U) DBA staining in normal canine intestinal mucosa used as positive control, DBA, 200×.

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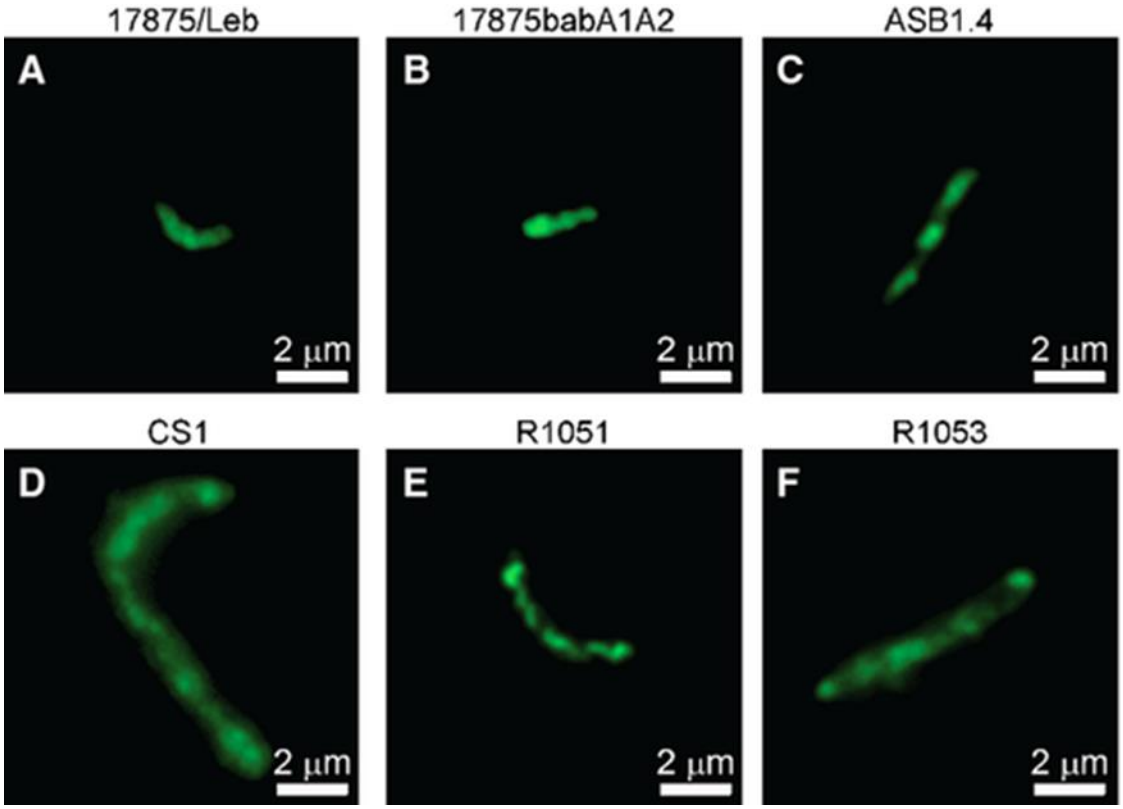

Figure 3. The panel shows the various morphologies of fluorescein - labeled Helicobacter spp. strains: (A) H. pylori 17875/Leb (B) H. pylori 17875babA1A2 (C) H. heilmannii s.s. ASB1.4 (D) H. felisCS1 (E) H. bizzozeroniiR1051 (F) H. salomonisR1053. Magnification 1000×.

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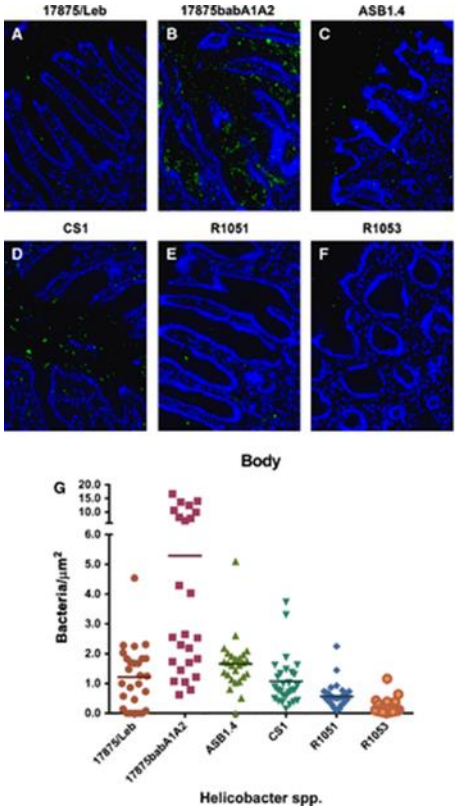

Figure 4. Evaluation of Helicobacter adherence to the body region of the canine gastric mucosa. Adhesion of fluorescein - labeled H. pylori model strains: (A) 17875/Leb and (B) 17875babA1A2; Adhesion of fluorescein - labeled Non - H. pylori Helicobacters (NHPH) strains: (C) ASB1.4, (D) CS1, (E) R1051and (F) R1053. Magnification 200×. (G) Quantification of adhesion of the different Helicobacter spp. strains. Each symbol represents the mean value obtained by evaluating at least five different fields within the foveolar or the glandular regions, as defined in 2. The differences of the mean values between the groups were statistically significant using one - way ANOVA ($p < .0001$).

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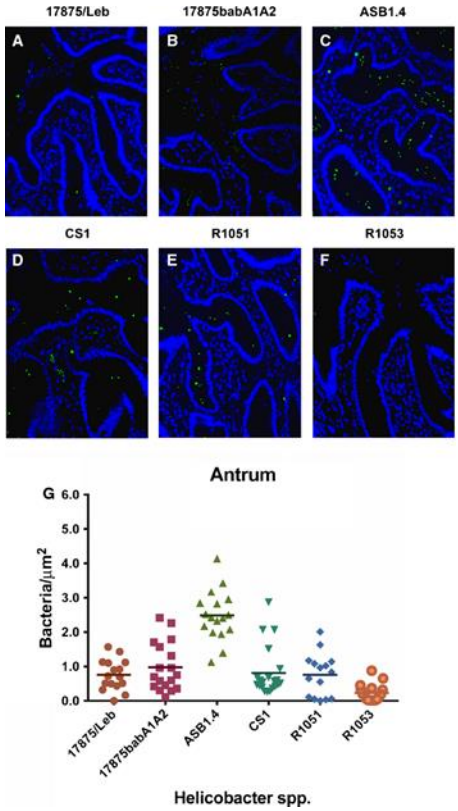

Figure 5. Evaluation of Helicobacter adherence to the antral region of the canine gastric mucosa. Adhesion of fluorescein - labeled H. pylori model strains: (A) 17875/Leb and (B) 17875babA1A2; Adhesion of fluorescein - labeled Non - H. pylori Helicobacters (NHPHs) strains: (C) ASB1.4, (D) CS1, (E) R1051 and (F) R1053. Magnification 200×. (G) Quantification of adhesion of the different Helicobacter spp. strains. Each symbol represents the mean value obtained by evaluating at least five different fields within the foveolar or glandular regions, as defined in 2. The differences of the mean values between the groups were statistically significant using one - way ANOVA ($p < .0001$).

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TABLES

Table 1 Antibodies used in immunohistochemistry

Monoclonal antibody	Antigen	Supplier/ References	Dilution
CA3F4	Le^a	Young et al. [55]	1 : 5
BG6	Le^b	Signet© (Dedham, MA, USA)	1 : 50
CA19-9	Sialyl-Le^a	Santa Cruz© (Santa Cruz, CA, USA)	1 : 500
SH1	Le^x	Fukushi et al. [56]	1 : 5
KM93	Sialyl-Le^x	Calbiochem© (Darmstadt, Germany)	1 : 60
AH6	Le^Y	Abe et al. [57]	1 : 10

Table 2 Terminal α-GalNAc (DBA), type 1 and type 2 Lewis antigens expression in canine gastric mucosa

			Type 1			Type 2		
		DBA	Le^a	Le^b	sLe^a	Le^x	Le^Y	sLe^x
Body (n = 8)	Positive immunostaining	8/8	1/8	0/8	0/8	5/8	8/8	4/8
	Immunostaining distribution							
	Sparse	0	1^{+}	0	0	0	0	0
	Focal	0	0	0	0	0	0	0
	Multifocal	0	0	0	0	$5^{+/++}$	$4^{+/++}$	$4^{+/++}$
	Generalized	$8^{+/++}$	0	0	0	0	4^{++}	0
Antrum (n = 6)	Positive immunostaining	6/6	0/6	0/6	0/6	6/6	6/6	1/6
	Immunostaining distribution							
	Sparse	0	0	0	0	0	0	1^{++}
	Focal	0	0	0	0	0	0	0
	Multifocal	0	0	0	0	$4^{++/+++}$	0	0
	Generalized	$6^{+/++}$	0	0	0	$2^{+/++}$	$6^{++/+++}$	0

DBA, *Dolichus biflorus* agglutinin.
Intensity variation observed: +weak; ++moderate; +++intense.

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