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## Glycoconjugate Binding of Gastric and Enterohepatic *Helicobacter* spp.

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***Helicobacter pylori* is able to utilize several lectin-like, protein-carbohydrate interactions for binding to mucins, cell surfaces, and extracellular matrix proteins. As determined by hemagglutination assays and binding of radiolabeled bacteria to glycosphingolipids on thin-layer chromatograms, strains of gastric helicobacters and enterohepatic helicobacters, including *Helicobacter canis*, *Helicobacter hepaticus*, and *Helicobacter bilis*, also demonstrated evidence for the presence of lectin-hemagglutinin adhesins. In addition, in *H. hepaticus* and *H. bilis*, binding may be sialic acid dependent. The presence or absence and differences in the levels of activity of lectin adhesins may reflect the species' ecological niche.**

The helicobacter genus includes more than 20 adequately characterized species to date (23). These species have been isolated from a number of hosts, including primates, pigs, cats, dogs, poultry, and rodents (23; <http://www.infek.lu.se/bakt/english/helicobacter.html>). Moreover, within their hosts, *Helicobacter* spp. have been identified from both the gastric and enterohepatic niches of the gastrointestinal tract, where they have been associated with a wide spectrum of clinical outcomes (5, 21). *Helicobacter pylori* is the most widely studied species of the genus and is associated with gastric pathology (9). The majority of *H. pylori* cells remain free swimming within the gastric mucosa. However, a minority of the population attach to the gastric epithelial surface and these play a vital role in the maintenance of infection (13). In addition, the population of *H. pylori* cells which attach may sporadically enter and survive within gastric epithelial cells and play a role in therapeutic failure (1, 15). Studies of many mucosal-surface-associated pathogens, including *H. pylori*, have shown that they utilize lectin-like adhesins on their surfaces to bind to glycoconjugate receptors in the mucin layer and on the epithelial surface (17, 27). Such lectins have been studied exhaustively in the case of *H. pylori* (16–18, 26). In particular, the bacterium has the noted ability to attach to both Lewis (Le)<sup>b</sup> and sialyl-dimeric-Le<sup>x</sup> antigens, which may be extremely relevant in the maintenance of a chronic infection (10, 20). However, little or no data exist regarding the potential adhesion strategies of other gastric and enterohepatic helicobacters. The aim of our study was to define lectin interactions for a range of gastric and enterohepatic helicobacters.

*H. pylori* strains (all human isolates) from the Culture Collection of the University of Gothenberg (CCUG), namely, CCUG 17874 and CCUG 17875 as well as the clinical isolate 119/95 from Lund University Hospital, were used. Gastric species examined in the present study included *Helicobacter mustelae* ferret isolates from the National Collection of Type Cul-

tures and the CCUG, including NCTC 12198/CCUG 25175 (equivalent strains), CCUG 23950, and CCUG 23951. “*Helicobacter suncus*” was originally isolated from the Japanese mouse shrew (6), and *Helicobacter felis* CCUG 28539 was isolated from a cat. Also, a human gastric clinical isolate initially identified as “*Helicobacter heilmannii*” was used (2). “*H. heilmannii*” organisms are a morphometry-based group and may contain a number of taxa, including *Helicobacter bizzozeronii*. Subsequent to the performance of our study, it was reported that the isolate used in the present study has been more definitively identified as *H. bizzozeronii* R-53 (11), and this designation is used here. A similar situation arises with the enterohepatic grouping of strains designated “*Helicobacter rappini*”/“*Flexispira rappini*,” which can include many taxa. The strain in the present study is a canine isolate from the University of Helsinki and is referred to as *Helicobacter* sp. *flexispira* taxon KT0201 here. Other enterohepatic helicobacters from various hosts were purchased from the CCUG, including *Helicobacter canis* CCUG 33835 (dog isolate), *Helicobacter bilis* CCUG 38995 (mouse isolate), *Helicobacter hepaticus* CCUG 33637 (mouse isolate), *Helicobacter fennelliae* CCUG 18820 (human isolate), and *Helicobacter pullorum* strains CCUG 33837 (chicken isolate), CCUG 33839 (human isolate), and NCTC 12827 (human isolate).

All helicobacters were tested in a hemagglutination (HA) assay by mixing equal volumes (30  $\mu$ l) of a bacterial suspension ( $10^9$  CFU ml<sup>-1</sup>) with a 0.75% (vol/vol) solution of washed erythrocytes in phosphate-buffered saline (PBS; pH 7.2). We used erythrocytes from horses, sheep, goats, rabbits, guinea pigs, hens (State Veterinary Institute, Uppsala, Sweden), and humans of blood types O and AB (blood bank, University Hospital Lund) (18). Alternatively, erythrocytes were mixed with PBS as a negative control. In cases of positive binding, the HA titer was determined, with 1 HA unit being defined as the minimum amount of bacterial cells required to cause complete HA.

An HA inhibition assay utilized glycoproteins (10 mg ml<sup>-1</sup>), including fetuin,  $\alpha_1$ -acid glycoprotein (orosomucoid), and asialofetuin (Sigma Chemical Co., St. Louis, Mo.), with the first two expressing terminal sialic acids which are important in the lectin activities of certain strains of *H. pylori*. Other terminal

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TABLE 1. HA patterns of gastric and enterohepatic helicobacters with native erythrocytes

Bacterial type and species	Reaction <sup>a</sup> with native erythrocytes from:						
	Human <sup>b</sup>	Horse	Sheep	Goat	Guinea pig	Hen	Rabbit
<b>Gastric</b>							
<i>H. mustelae</i> NCTC12198	+	+	+	+	+	+	+
<i>H. pylori</i> CCUG 17874 <sup>c</sup>	+	+	+	ND	+	ND	+
<i>H. pylori</i> 119/95	+	+	+	+	+	+	+
<i>H. bizzozeronii</i> R53	+	+	+	+	+	+	+
" <i>H. suncus</i> "	+	+	—	+	+	+	+
<i>H. felis</i> CCUG 28539	—	—	—	—	—	—	+
<b>Enterohepatic</b>							
<i>H. canis</i> CCUG 33835	—	—	—	—	—	—	+
<i>H. bilis</i> CCUG 38995	+	+	+	—	+	—	+
<i>H. hepaticus</i> CCUG 33637	±	±	±	—	±	—	+
<i>H. fennelliae</i> CCUG 18820	—	—	—	—	—	—	+
<i>Helicobacter</i> sp. flexispira taxon KT0201	—	—	—	—	—	—	+
<i>H. pullorum</i> NCTC 12827	—	—	—	—	—	—	+
<i>H. pullorum</i> CCUG 33837	—	—	—	—	—	—	+
<i>H. pullorum</i> CCUG 33839	—	—	—	—	—	—	+

<sup>a</sup> HA reaction in U-shaped wells, determined visually. +, a carpet of erythrocytes; —, a dot of cells; ±, occasional positive results for binding; ND, not determined.

<sup>b</sup> Results for AB and O blood groups were similar.

<sup>c</sup> See reference 18.

monosaccharide residues predominate in the case of asialofetuin, including galactose, mannose, and *N*-acetylgalactosamine. In addition, gastric mucin fractions of human and porcine origins were also used in HA inhibition as described previously (18). Gastric mucins are rich in fucose, galactose, *N*-acetylgalactosamine, *N*-acetylglucosamine, and terminal sialic acid. All assays were carried out at least in triplicate.

Two broad HA profiles were observed (Table 1). *H. pylori* 119/95, *H. mustelae* NCTC 12198, *H. bizzozeronii*, "*H. suncus*," and *H. bilis* CCUG 38995 caused HA for all or the majority of erythrocytes tested (five to seven of seven kinds). Variable HA activity was noted for *H. hepaticus*, although in the majority of tests, no binding was observed. In contrast, most enterohepatic helicobacters and *H. felis* CCUG 28539 did not cause the HA of any erythrocytes tested, with the exception of rabbit blood cells. *H. mustelae*, *H. pylori*, *H. bizzozeronii*, and *H. bilis* were analyzed in more detail for their precise HA titers (Table 2). The HA of rabbit erythrocytes could be largely inhibited by orosomucoid. However, human and porcine mucin preparations proved to be poor inhibitors for HA. In most cases, asialofetuin proved to be more effective than fetuin in inhib-

iting HA, except with *H. bilis* CCUG 38995, for which the converse was true (data not shown). Subsequently, both fetuin and asialofetuin were covalently coupled to carboxylate-modified latex particles (diameter, 0.8 µm; Seradyn, Indianapolis, Ind.) as described previously (17). Bacterial suspensions (10<sup>9</sup> CFU ml<sup>-1</sup>) were mixed with equal volumes (10 µl) of coupled latex beads on a glass slide and tilted for 30 s, and results were assessed visually. Both gastric and enterohepatic helicobacters demonstrated reactivity with fetuin- and asialofetuin-coated beads. However, *H. felis* CCUG 28539 failed to react with fetuin or asialofetuin and reactions with all *H. pullorum* isolates were weak. Furthermore, *H. bilis* CCUG 38995 agglutinated more strongly with these beads than any other enterohepatic helicobacter.

Erythrocytes were treated with neuraminidase in order to remove terminal *N*-acetylneuraminic (sialic) acid and expose other potential carbohydrate binding sites (8, 16). The principal effect of neuraminidase treatment of goat erythrocytes was a decrease in the titers of *H. pylori* 119/95 and *H. bilis* CCUG 38995 (Table 2). Neuraminidase treatment of rabbit erythrocytes had a strain-specific effect on titer.

HA was also decreased or eliminated following proteolytic treatments of the bacteria using either proteinase K (1 mg ml<sup>-1</sup>, 65°C, 1 h), pronase E (1 mg ml<sup>-1</sup>, 37°C, 1 h), or heat treatment (100°C for 5 min). In order to determine the localization of the factors involved in positive HA results, an acid glycine extract from the bacteria was tested. Acid-glycine extracts have been routinely used to characterize the cell surface of *H. pylori*, and this fraction contains predominantly surface-associated proteins with minor amounts of cytoplasmic proteins and lipopolysaccharide. Proteins were mildly extracted using a 0.2 M acidic glycine (pH 2.2) wash for 15 min at 20°C (19). The use of acid-glycine extracts in both the HA and the particle agglutination assay yielded results identical to those for whole bacterial cells.

The binding of *Helicobacter* spp. to both acidic and nonacidic fractions of glycosphingolipids was subsequently assessed. Gly-

TABLE 2. HA titers for HA-positive species in native and neuraminidase-treated goat and rabbit erythrocytes

Bacterial type and species	HA titer <sup>a</sup>			
	Goat erythrocytes		Rabbit erythrocytes	
	Native	Neuraminidase treated	Native	Neuraminidase treated
<b>Gastric</b>				
<i>H. mustelae</i> NCTC 12198	2	2	8	>1,024
<i>H. pylori</i> 119/95	8	4	8	1
<i>H. bizzozeronii</i> R-53	1	1	>1,024	>1,024
<b>Enterohepatic</b>				
<i>H. bilis</i> CCUG 38995	8	2	1	2

<sup>a</sup> HA titers were determined with 1 HA unit being defined as the minimum amount of bacterial cells required to cause complete HA.

TABLE 3. Binding of <sup>35</sup>S-labeled *Helicobacter* spp. to glycosphingolipids on thin-layer chromatograms<sup>a</sup>

Trivial name	Structure <sup>b</sup>	Binding <sup>c</sup> of glycosphingolipids to:							
		Gastric species				Enterohepatic species			
		<i>H. mustelae</i> 25715	<i>H. mustelae</i> 23950/1	<i>H. pylori</i> 17874	<i>H. pylori</i> 17875	<i>H. felis</i> 28539	<i>H. canis</i> 33835	<i>H. bilis</i> 38995	<i>H. hepaticus</i> 33637
Lactosylceramide	Galβ4Glcβ1Cer	+	+	+	+	+	+	+	+
Isoglobotri	Galα3Galβ4Glcβ1Cer	+	+	+	+	+	+	+	+
Gangliotetra	Galβ3GalNAcβ4Galβ4Glcβ1Cer	+	+	+	+	+	+	+	+
Le <sup>a</sup> -5	Galβ3(Fucα4)GlcNAcβ3Galβ4Glcβ1Cer	—	—	—	—	—	—	—	—
Le <sup>b</sup> -6	Fucα2Galβ3(Fucα4)GlcNAcβ3Galβ4Glcβ1Cer	—	—	—	+	—	—	—	—
Globotetra	GalNAcβ3Galα4Galβ4Glcβ1Cer	—	—	—	—	—	—	—	—
Lactotetra	Galβ3GlcNAcβ3Galβ4Glcβ1Cer	+	+	+	+	+	+	+	+
Acid glycosphingolipids of human granulocytes		±	—	+	—	—	—	—	+

<sup>a</sup> Identical results were noted for agar- and broth-grown bacteria.<sup>b</sup> "Cer", ceramide composition; Fuc, fucose composition.<sup>c</sup> Binding is defined as follows: + denotes a significant darkening on the autoradiogram when 2 μg (or 40 μg in the case of the last sample) was applied to TLC plates, — denotes no binding, and ± denotes occasional positive results for binding.

cosphingolipids were isolated by standard procedures (12). The identities of the purified glycosphingolipids were confirmed by mass spectrometry (22), proton nuclear magnetic resonance spectroscopy (14), and degradation studies (24, 29).

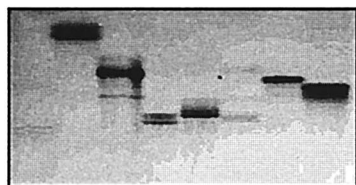
Mixtures of glycosphingolipids (40 μg/lane) or pure compounds (2 μg/lane) were subsequently separated by thin-layer chromatography (TLC) on glass- or aluminum-backed Silica Gel 60 high-performance TLC plates (Merck, Darmstadt, Germany), with chloroform-methanol-water (60:35:8, by volume) as the solvent system. Chemical detection was accomplished with anisaldehyde (28). For labeling, colonies were inoculated on Brucella agar plates, and 50 mCi of [<sup>35</sup>S]methionine (Amersham, Little Chalfont, United Kingdom) diluted in 0.5 ml of PBS, pH 7.3, was sprinkled over the plates. Alternatively, colonies were inoculated (10<sup>5</sup> CFU ml<sup>-1</sup>) in Ham's F-12 medium (Gibco BRL, Paisley, United Kingdom), with 10% heat-inactivated fetal calf serum and 50 mCi of [<sup>35</sup>S]methionine per 10 ml of medium. After incubation under standard conditions, the cells were harvested, washed, resuspended to 10<sup>8</sup> CFU ml<sup>-1</sup> in PBS, and incubated with shaking under microaerophilic conditions at 37°C for 12 to 24 h. Both labeling procedures resulted in suspensions with specific activities of approximately 1 cpm per 100 *H. pylori* organisms (3). Binding of the labeled bacteria to glycosphingolipids separated by TLC was achieved

using a bacterial-overlay technique coupled with autoradiography detection using XAR-5 X-ray films (Eastman Kodak, Rochester, N.Y.) as described elsewhere (7).

It has been established previously that both *H. pylori* and *H. mustelae* bind gangliotetraosylceramide (5a), which was confirmed in this study; a similar capacity was also demonstrated for *H. felis* CCUG 28539, *H. canis* CCUG 33835, and *H. hepaticus* CCUG 33637 (Table 3). Furthermore, in common with *H. pylori*, we found that both the gastric and enterohepatic *Helicobacter* spp. tested were capable of binding to lactotetraosylceramide, lactosylceramide with phytosphingosine, and/or hydroxy fatty acids and isoglobotriaosylceramide. In contrast, within the strains and species tested, binding to Le<sup>b</sup> glycosphingolipid was solely observed for *H. pylori* CCUG 17875 (Table 3).

The binding of certain *H. pylori* strains to slow-migrating gangliosides in the acid glycosphingolipid fraction of human granulocytes is sialic acid dependent, and this fraction was therefore used as an indicator of sialic acid recognition. Binding to this fraction was noted for *H. hepaticus* CCUG 33637 (exemplified in Fig. 1B, lane 1) and *H. pylori* CCUG 17874 and occasionally for *H. mustelae* CCUG 25715 (Table 3). It should be noted that no differences were observed in the levels of binding to glycosphingolipids of the agar- and broth-grown

## A. Chemical detection



1 2 3 4 5 6 7 8

B. *H. hepaticus* 33637

1 2 3 4 5 6 7 8

C. *H. bilis* 38995B

1 2 3 4 5 6 7 8

FIG. 1. (A to C) Chemical detection of glycosphingolipids (A) and chromatogram assay detection of their binding to *Helicobacter hepaticus* (B) and *Helicobacter bilis* (C). Lanes (where Cer is ceramide and Fuc is fucose): 1, acid glycosphingolipid fraction of human granulocytes, 40 μg; 2, Galβ4Glcβ1Cer (lactosylceramide) of dog intestine, 2 μg; 3, Galα3Galβ4Glcβ1Cer (isoglobotriaosylceramide) of dog intestine, 2 μg; 4, Galβ3GalNAcβ4Galβ4Glcβ1Cer (gangliotetraosylceramide) of mouse feces, 2 μg; 5, Galβ3(Fucα4)GlcNAcβ3Galβ4Glcβ1Cer (Le<sup>a</sup>-5 glycosphingolipid) of human meconium, 2 μg; 6, Fucα2Galβ3(Fucα4)GlcNAcβ3Galβ4Glcβ1Cer (Le<sup>b</sup>-6 glycosphingolipid) of human meconium, 2 μg; 7, GalNAcβ3Galα4Galβ4Glcβ1Cer (globotetraosylceramide) of human erythrocytes, 2 μg; 8, lactotetraosylceramide (Galβ4GlcNAcβ3Galβ4Glcβ1Cer) of human meconium, 2 μg.



bacteria used in this study, in contrast to what occurred with the polyglycosylceramide binding of *H. pylori*.

The ability of predominantly gastric and also certain enterohepatic *Helicobacter* spp. to both hemagglutinate erythrocytes and bind to glycosphingolipids is indicative of the use of host-carbohydrate binding by these species in their adhesion strategies. The elimination of HA following proteolytic and heat treatment characterizes these interactions with erythrocytes as protein-carbohydrate or lectin based. In addition, the ability of cell surface acid-glycine extracts and whole cells to yield identical levels of HA implies that this lectin-like activity is not dependent on viable bacteria and, not surprisingly for putative adhesins, that it is likely to be present on the bacterial surface. Such adhesins have been described previously for *H. pylori* and have been classified as either sialic acid dependent or independent (8, 18). The decrease in HA titer following neuraminidase treatment of goat erythrocytes suggests a role for sialic acid in the binding of strains of *H. bilis* and *H. pylori*. It has been established that horse and rabbit erythrocytes have comparatively low levels of sialic acid compared with those of the other erythrocytes tested (4). In addition, the structures and composition of sialic acid may differ among the types of erythrocytes (4). The lower HA titer for *H. bilis* noted for native rabbit erythrocytes is consistent with these data. However, the identical HA titers for *H. pylori* 119/95 for horse and rabbit erythrocytes suggests the ability of this strain to also use sialic acid-independent binding. In addition, the presence of a sialic acid-dependent adhesin on the surface of *H. bilis* was further illustrated by the ability of the bacterium to strongly bind fetuin, which was also a more potent inhibitor of its HA activity than asialofetuin.

However, sialic acid recognition as evidenced by binding to the acid glycosphingolipid fraction of human granulocytes was observed for strains of *H. hepaticus* (Fig. 1B, lane 1) and *H. pylori* but was not observed for *H. bilis* (Fig. 1C, lane 1). Differences noted in the two methods used to characterize the protein-carbohydrate interactions in particular for strains of *H. hepaticus* and *H. bilis* may have been due to phase or strain variation in the levels of expression of these adhesins or the presence of a mixed culture of strains. Both hypotheses are supported by our observation of occasional binding for *H. hepaticus* CCUG 33637 and *H. mustelae* CCUG 25715 in the HA and overlay assays, respectively. More-sensitive techniques to detect binding, including surface plasmon resonance, may improve the detection of such lectin-like activity.

A further possibility is that the putative bacterial lectins have different fine specificities. It is known that human erythrocytes contain a number of gangliosides which may be involved in lectin interactions. Their HA profiles and their ability to bind to various nonacid glycosphingolipids from human erythrocytes suggest that gastric helicobacter strains are capable of recognizing heterogenous glycoconjugate receptors but that the majority of enterohepatic helicobacters and *H. felis* may have a more defined set of adhesins which do not seem to rely on sialic acid. Alternatively, it has been previously established that different gastric helicobacter isolates from dogs, including *H. felis* and *H. bizzozeronii*, differ in morphology and perhaps also motility, which may in turn have an impact on adhesion (25). A similar situation may arise in relation to the enterohepatic species. However, as demonstrated with *H. pylori*, the

expression of sialic acid-dependent binding may be strain specific or due to phase variation as noted by Mahdavi and colleagues (20). Thus, the low numbers of strains of different helicobacter species, in particular for the enterohepatic species, represent a limitation in the present study that awaits better methods of isolation, culture, transport, and resuscitation for *Helicobacter* spp.

The differential levels of expression of lectins on the surfaces of helicobacters discerned in our study may reflect the bacterium's niche and/or ability to strongly adhere to the host's cells. As was suggested recently, a varied lectin-based adhesion strategy may be useful at specific instances during the course of a chronic infection, as changes occur in the levels of receptors expressed in inflamed tissue (20). From the present study it is apparent that certain hepatobiliary helicobacters, namely, *H. hepaticus* and *H. bilis* may share common adhesion strategies with *H. pylori*. The pathogenic potential of similar adhesins and other common pathogenic factors, in particular, urease, for these hepatic helicobacters is as yet unknown. Further characterization of the effects of these putative adhesins in the variations of particular strains would be important to gain insights into the different adherence strategies utilized by these emerging pathogens but requires a greater availability of fresh clinical isolates of these species.

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