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Effect of heparin, fucoidan and other polysaccharides on adhesion of enterohepatic *Helicobacter* species to murine macrophages

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Abstract

Helicobacter species have been isolated and cultured from both the gastric and enterohepatic niches of the gastrointestinal tract, and are associated with a wide spectrum of diseases. Some members of enterohepatic *Helicobacter* species (EHS) include *H. bilis, H. hepaticus* and *H. pullorum* are associated with chronic inflammatory and proliferative bowel inflammation, hepatitis, and in experimental murine studies with hepatic cancer. The present study aimed to explore if poly-sulphated polysaccharides can prevent adhesion of EHS to the murine macrophage cell line J774A.1. A competitive binding assay showed that heparin and heparan sulphate at a concentration of 1.25 mg/ml reduced binding of *H. hepaticus* and *H. pullorum* to the host cells, but not *H. bilis.* Of tested *Helicobacter* spp the highest inhibition by heparin was demonstrated for *H. pullorum* ($P<0.01$), the most hydrophilic strain. Partially or completely de-sulphated heparin derivates lost the ability to inhibit adherence of EHS, indicating the importance of sulphated groups of heparin. The most efficient inhibitor of EHS binding to macrophages was fucoidan, which reduced bacterial adhesion of the three enterohepatic *Helicobacter* species to a greater extent than heparin, 60-90% inhibition vs 30-70% inhibition by heparin. Identification of receptors that EHS ligands bind to is important for understanding the development of infection, and may provide a rational target to prevent infection and therapy.

Introduction

Many pathogenic bacteria and viruses use cell surface glycosaminoglycans (GAGs) and proteoglycans as cell and tissue binding molecules. To cause infection, bacteria adhere to target cells and colonize the cell surfaces. Microbial adherence to the host cell surfaces is commonly initiated by non-specific hydrophobic and electrostatic interactions, followed by a second step involving carbohydrate specific lectin-like interactions [1,2]. Prevention of such adhesion is an attractive target for the development of new therapies against infection. Soluble carbohydrates recognized by the bacterial surface lectins block the adhesion of the bacteria to animal cells *in vitro* [3]. Various sulphated polysaccharides (heparin, heparin oligosaccharides and fucoidan) competitively inhibited the gastric pathogen *H. pylori* binding of heparan sulphate [4,5]. However, it has not been studied if heparin and other polysaccharides inhibit binding of non-gastric, enterohepatic *Helicobacter* species (EHS) to the host cells. One report described glycosphingolipid binding specificity of the enterohepatic *H. hepaticus* species [6]. Thus, data are sparse about possible anti-adhesive agents to protect host cells from enterohepatic *Helicobacter* species infection.

We have now explored whether polysulphated polysaccharides can prevent adhesion of EHS to host cells and analyzed if such polysaccharides might act as an anti-adherent agent.

Materials and methods

Bacterial strains

H. bilis (CCUG 38995), *H. hepaticus* (CCUG 33637) and *H. pullorum* (CCUG 33838) as well as *H. pylori* strain (CCUG 17875) were obtained from the Culture Collection, University of Gothenburg, Sweden (www.ccug.gu.se). The EHS were grown for three to four days on Brucella agar supplemented with 10% horse serum, 5% horse blood, 1% Vitox, 1% haemin under microaerophilic conditions at 37°C (Anoxomat, MART®, Lichtenvoorde, The Netherlands). *H. pylori* was cultured for two days on Gab-Camp agar without antibiotics in a humidified athmosphere of 5% CO_2 at 37 °C [7,8].

Chemicals

Heparin sodium salt from porcine intestinal mucosa, fucoidan and dextran sulphate sodium salt were purchased from Fluka (Buchs, Switzerland), heparin monosulphate sodium salt from Chemos GmbH (Regenstauf, Germany), De-N-sulphated heparin sodium salt, N-acetyl-de-O-sulphated heparin sodium salt, chondroitin sulphate B sodium salt, and dermatan sulphate, poly (4-styrenesulfonic acid) (PSS) from Sigma-Aldrich (St. Louis, MO, USA). Molecular structures of the substances are listed in Table 1.

Labelling of bacteria

Bacteria were labelled with fluorescein isothiocyanate (FITC) (Sigma-Aldrich) as earlier described [9] except in this study live bacteria were used. Briefly, bacteria were incubated with FITC, 100 µg/ml in carbonate buffered saline containing 1% of dimethyl sulfoxide (DMSO) for 30 min at 22°C and adjusted to an optical density of 1.0, wave length 540 nm

corresponding to 10^8 colony forming units (CFU).

Host cell culture

The murine macrophage cell line J774A.1 (ATCC, Vanassas, VA, USA) (www.lgcpromochem.com/atcc) was cultured in RPMI 1640 medium with L-glutamine (Gibco, Paisley, Scotland, UK) supplemented with 10% of fetal bovine serum (FBS, Gibco) and 50 μg/ml gentamicin sulphate (Gibco) for 2-3 days at 37 °C in a humidified 5% CO_2 atmosphere. Before stimulation cell were seeded into an 8-well chamber slide (Nalge Nunc Int., Naperville, IL, USA) with 5×10^5 cells per well and incubated for 20-24 h. The cells were tested negative for mycoplasma contamination (DAPI, Gibco). Viability of macrophages was assessed by trypan blue staining and was consistently more than 95%.

Competitive binding adhesion assay

According to previously published data, heparin showed optimal inhibition of *H pylori* adhesion at a concentration of 1 mg/ml [10]. FITC labelled bacterial suspensions was mixed with equal volumes of RPMI alone or with polysaccharides at a concentration of 1.25 mg/ml, agitated for 1 h at 37°C and added to a macrophage monolayer in relation 50 CFU per one macrophage.

Fluorescence microscopy

J774A.1 macrophage monolayers were inoculated with bacteria and incubated at 37 °C in a microaerophilic athmosphere for 30 min, washed six times in PBS (phosphate buffered saline pH 7.2) , and fixed with 3.7% formaldehyde in PBS for 10 min. Before staining with rhodamine-phalloidin (Sigma-Aldrich) (3 μg/ml in PBS), cells were permeabilized by using absolute acetone at –20°C for 3 min, washed with PBS and incubated with rhodamine

phalloidin for 30 min at 22°C. After washing with PBS, cover slips were placed on slides in glycerol mounting medium (Euroimmun, Lübeck, Germany).

Cell surface hydrophobicity test

The salt aggregation test (SAT) was performed as previously described [11]. Ten µl of the bacterial suspension were mixed with 10 µl of ammonium sulphate $(NH_4)_2SO_4$) pH 6.8, of various molarities (0.02, 0.2, 0.8, 1.6, 3.0, 4.0 M) on a glass slide. The lowest concentration of ammonium sulphate at which bacterial aggregation was visible within 2 min was scored as the SAT value.

Statistical analysis

Experiments were performed in triplicates in two independent sets, differences between medians were determined by the Mann-Whitney U test, and considered significant at $P < 0.05$. Statistical analysis was done using the GraphPad InStat 3 software.

Results

Competitive inhibition of EHS adhesion to phagocytes by polysulphated polysaccharides

In the present study we chose the murine macrophage cell line J774A.1 since *H. pylori* was shown to adhere strongly to these cells [9,10]. The macrophages were inoculated with EHS pretreated with different polysulphated agents (heparin, heparan sulphate, chondroitin sulphate, dextran sulphate and fucoidan). The *H. pylori* strain served as a positive control for bacterial adhesion to host cells and for the carbohydrate competitive binding assay.

According to a previously described method [9] each of the polysaccharides was added to FITC-labeled bacteria before inoculation of host cells. The concentration that showed the

optimal inhibition of bacterial attachment to host cells was found to be 1.25 mg/ml final concentration (data not shown).

By blocking the GAG-binding sites on the bacterial surface with heparin or HS, a decrease in binding of the EHS to the macrophage cells was observed. Of the tested *Helicobacter* spp the highest inhibition by heparin was demonstrated for *H. pullorum* ($P<0.01$). Inhibition of *H. bilis* adhesion of the macrophages was next to significant (one-tailed $P<0.05$, two-tailed $P>0.05$). *H. hepaticus* adhesion was moderately reduced by heparin to 50% ($P<0.01$) and slightly reduced by HS to 37% ($P<0.05$) (Fig. 1a, b) while *H. pullorum* adhesion was notably reduced by both glycosaminoglycans by more than 75% ($P<0.01$) (Fig. 1c).

Another sulphated GAG, chondroitin sulphate B (ChS), was much less effective compared with heparin and HS (Fig. 1). For the three *Helicobacter* species tested, adherence to the J774A.1 macrophage cells was unaffected by pretreatment with the ChS, which might be explained by a lower density of sulphate groups per molecule of the ChS compared with heparin (Table 1). Heparan sulphate chains usually contain 0.8-1.4 sulphate groups per disaccharide unit while heparins synthesized in intracellular granulae of mast cells contain ≥ 2.4 sulphate groups per unit and ChS contains 1 sulphate group per disaccharide unit.

Homopolysaccharide DexS inhibited *H. bilis* cell adhesion by 25% ($P<0.05$) and moderate inhibition of *H. pullorum* binding approximately 50% ($P<0.05$).

The best inhibition of EHS tested was found with the fucoidan, 63-93% ($P<0.05$) (Fig. 1a, b, c, and Fig. 2), followed by heparin, HS and DexS. Fucoidan prepared from the *Fucus vesiculosus* is composed of 44.1% fucose and 26.3% sulfate.

De-sulphation of heparin reduced inhibition of EHS to macrophages

We investigated the effects of selectively N- and O-de-sulphated derivatives of heparin on the adhesion of EHS to macrophages. These derivatives of heparin lost their inhibitory activity compared to native heparin.

As mentioned before, adherence of *H. hepaticus* and *H. pullorum* were inhibited by the native heparin. Partially de-sulphated DeNhep was not as effective as heparin for *H. pullorum* (Fig. 3), whereas completely De-sulphated DeOhep lost ability to block *H. bilis* and *H. pullorum* adherence to host cells.

Hydrophobicity

Bacterial cell surface hydrophobicity estimated by the SAT showed that coccoid forms of *H. bilis* aggregated in 0.8 M ammonium sulphate while spiral forms of *H. bilis* as well as the other *Helicobacter* did not aggregate under this condition. Both these morphological forms of *H. hepaticus* auto-aggregated in 3 M ammonium sulphate, *H. pullorum* did not form clumps even in 4 M ammonium sulphate.

Discussion

Hydrophobicity and microbial adhesion to the host cells

Cell surface hydrophobicity (CSH) of bacteria often contributes to their adherence to host cells. A marked hydrophobicity was found to be related to an increased capacity for *Escherichia coli*, *Salmonella* species, and strains of *Shigella flexneri* to adhere to host cell [12,13]. In order to better understand the *Helicobacter* spp adhesion process to murine macrophages, we tested the *Helicobacter* strains by the SAT, *H. pullorum* was found to be the most hydrophilic strain studied.

The SAT results were compared with the macrophage adhesion assay, direct correlation between inhibition of microbial adherence by EHS and their hydrophobicity was observed. The most effective reduction of bacterial attachment to host cells by heparin, heparan sulphate and fucoidan was demonstrated for the highly hydrophilic strain *H. pullorum* whereas the same polysaccharides removed less *H. hepaticus* binding, which was found to be a more hydrophobic strain. Cell adhesion of *H. bilis*, the most hydrophobic strain, was effectively inhibited by fucoidan. The differences in inhibition by heparin of these strains might be explained by variations in their hydrophobicity.

Our results emphasize a possible importance of heparin and heparansulphate as specific anti-adhesive molecules for EHS towards host phagocytic cells. Adhesion of hydrophilic EHS (*H. hepaticus* and *H. pullorum*) to the J477A.1 cell line was inhibited by the addition of exogenous sulphated heparin, indicating that GAGs are possible receptors on the host phagocytic cells and have a higher capacity to inhibit bacterial adhesion to host cells compared with less sulphated GAG chondroitin sulphate B (dermatan sulphate) and the polysulphated homopolysacchride dextran sulphate. Heparin-dependent bacterial binding to macrophages was strongly dependent on sulphation, whereas partially de-sulphated heparin retained a low capacity to impair *Helicobacter* binding to host cells. Completely de-sulphated heparin did not inhibit bacterial-host cell binding, indicating that sulphation contributes to heparin involvement in the bacterial adhesion to host cells. Sulphation was even shown to be a structural requirement for specific binding of *C. trachomatis* to the host cell surface heparan sulphate proteoglycans [14].

Bacterial CSH can be considered as an additional factor in determining adherence properties of many microbes to host cell surfaces. The highest reduction of bacterial binding by polysulphates to host cells was achieved for *H. pullorum*, the most hydrophilic strain. Moderate inhibition of adherence was demonstrated for *H. hepaticus*, a more hydrophobic

strain, and finally, *H. bilis*, the most hydrophobic strain was not efficiently affected by heparin but by fucoidan only.

Adherence of *Helicobacter* species to host cells is an early step in the pathogenesis of microbial infection. Identification of the tissue receptors for enteric *Helicobacter* is important to understand the development of infection, and may provide a rational target to develop a new therapy concept. Oral immunization of BALB/c mice with a vaccine, composed of *H. pylori* heparan-sulphate binding proteins, prevented colonization of the gut mucosa by a mouse-adapted *H. pylori* strain, and reduced adhesion of the pathogen to the gastric mucosa to less than 10% in immunized mice [15]. Similar strategies were recently discussed to prevent papilloma virus infection (HPV) in the female genital tract to prevent HPV associated cervix cancer [16, 17].

Apart from a *Helicobacter* vaccine, a carbohydrate-based therapy aimed to detach adherent *Helicobacter* now deserves more attention. Such a therapeutic strategy by administrating polysaccharides with anti-adhesive properties, including heparin and fucoidan, may prove to be good candidates for a vaccine.

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Table 1 Molecular structure of polysaccharides tested for inhibition of enterohepatic *Helicobacter* adherence to macrophages.

Note: "NS" indicates position of OSO^{3-} group.

Studied molecule	**Structure of the main monomeric units**
Glycosaminoglycans (heteropolysaccharides)	
Heparin	-4IdoA(2S)α1-4GlcNS(6S)β1-
Heparan sulphate	-4GlcAβ1-4GlcNAcα1- (around 50% of all residues) -4IdoA(2S)α1-4GlcNS(6S)β1-
De-N-sulphated heparin	-4IdoA(2S)α1-4GlcN(6S)β1-
N-Acetyl-de-O-sulphated heparin	-4IdoAα1-4GlcNβ1-
Chondroitin-sulphate B	-4IdoAα1-3GalNAc(4S)β1-
Homopolysaccharides	
Dextran sulphate	-6Glc(+/-2S, 3S, +/-4S)α1-6Glc(+/-2S, 3S, +/-4S)α1- (around 2.3 sulphate groups per Glc)
Fucoidan	-3Fucα1-3Fuc(4S)α1-

Fig. 1

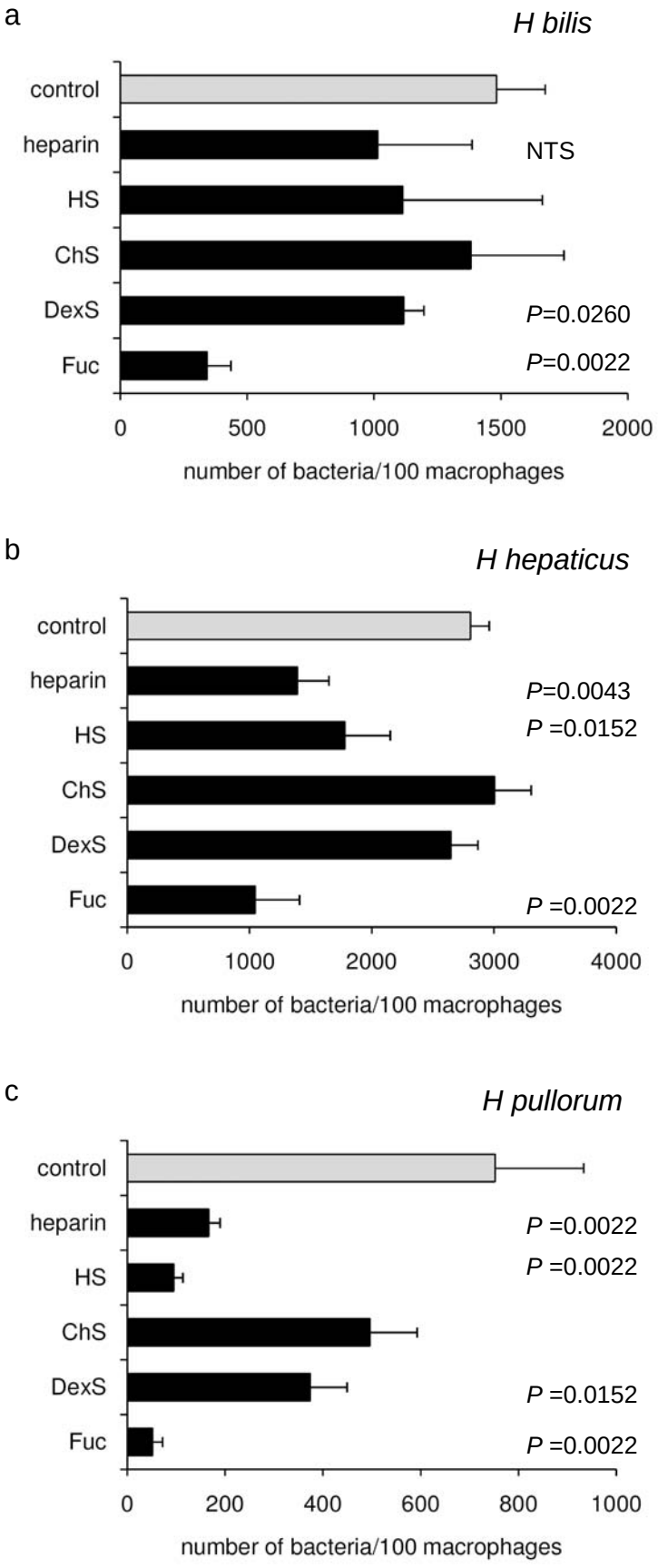

a
H bilis
control
heparin
HS
ChS
DexS
Fuc
NTS
P=0.0260
P=0.0022
0
500
1000
1500
2000
number of bacteria/100 macrophages
b
H hepaticus
control
heparin
HS
ChS
DexS
Fuc
P=0.0043
P =0.0152
P =0.0022
0
1000
2000
3000
4000
number of bacteria/100 macrophages
c
H pullorum
control
heparin
HS
ChS
DexS
Fuc
P =0.0022
P =0.0022
P =0.0152
P =0.0022
0
200
400
600
800
1000
number of bacteria/100 macrophages

Fig. 2

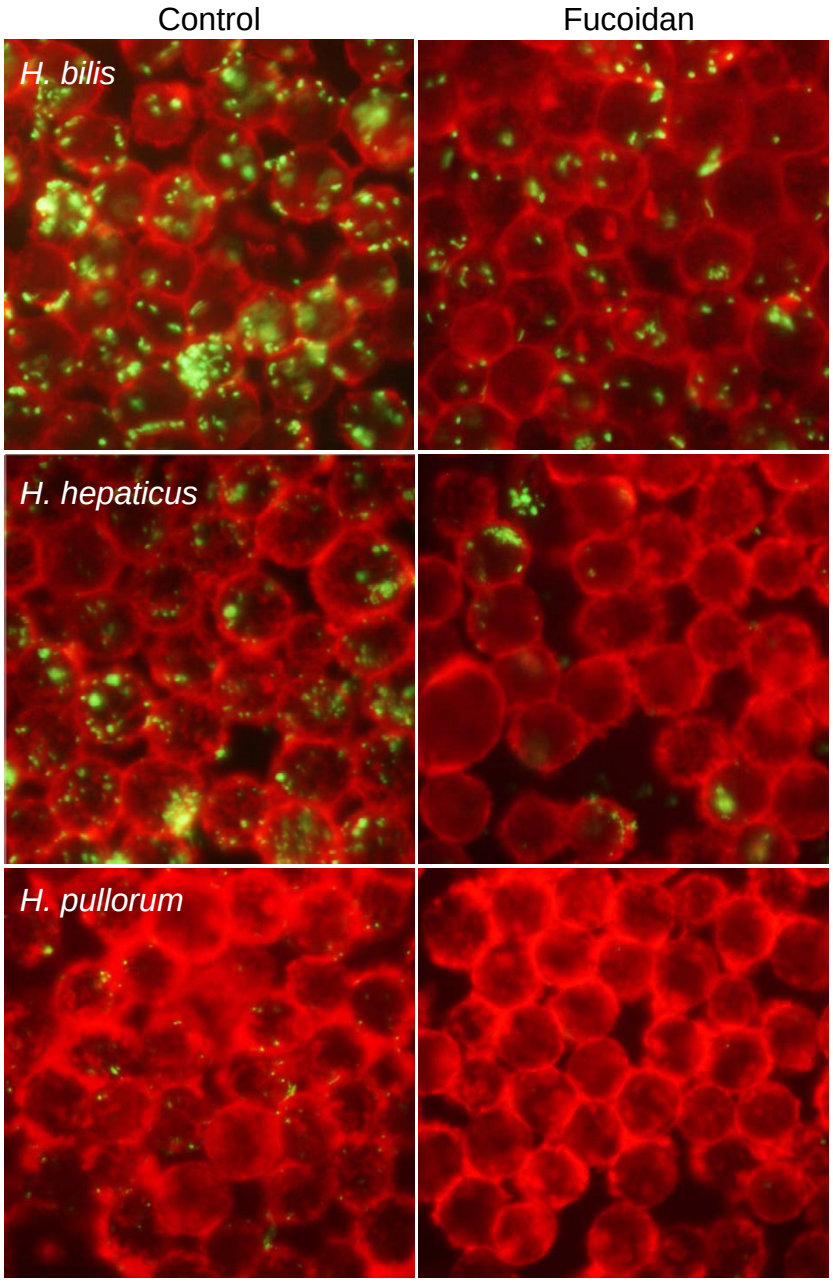
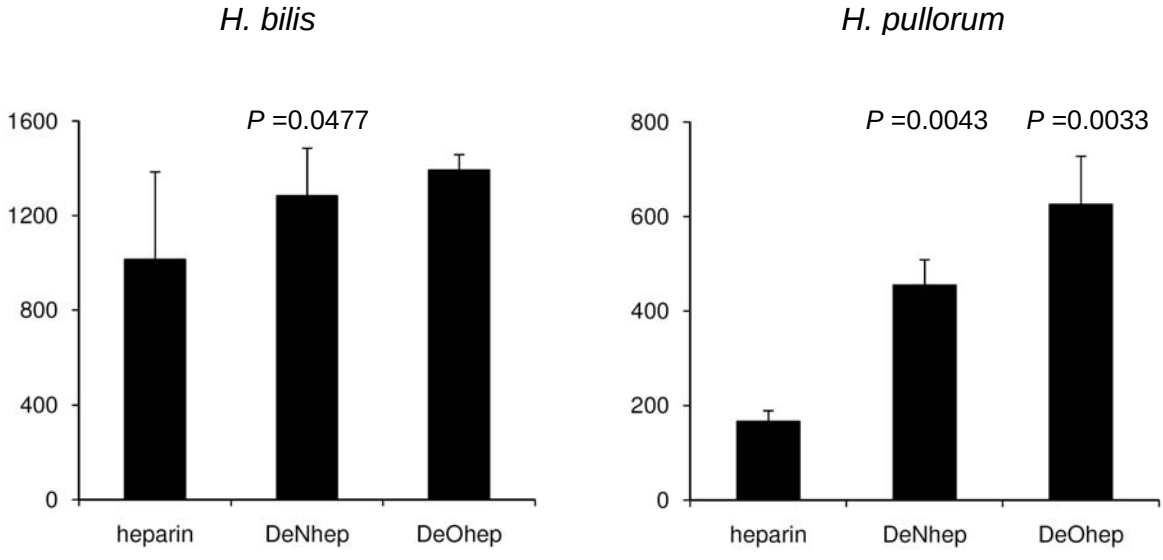


Fig. 3


H. bilis
H. pullorum
P =0.0477
P =0.0043
P =0.0033
1600
1200
800
400
0
800
600
400
200
0
heparin
DeNhep
DeOhep
heparin
DeNhep
DeOhep

Legends to figures

Fig. 1a, b, c. Adhesion of *H. bilis* (a), *H. hepaticus* (b) and *H. pullorum* (c) to macrophage cells after treatment with different polysaccharides. Heparin and heparan sulphate (HS) significantly reduced *H. hepaticus* and *H. pullorum* binding to macrophages whereas chondroitin sulphate (Ch) was not effective. Bars represent median ± MAD (Median absolute deviation). Note: NTS – next to significant, the *P*-values are for two-tailed tests against control.

Fig. 2. Inhibition of enterohepatic *Helicobacter* adhesion to macrophages by fucoidan from *Fucus vesiculosus.* Macrophages were stained with phalloidin-TRITC, *Helicobacter* species are directly labeled with FITC. Magnification × 500.

Fig. 3. Partially de-sulphated heparin (de-N-de-sulphated - DeNhep) and completely de-sulphated heparin (de-O-desulphated - DeOhep) were less effective for blocking microbial adhesion than native heparin. The most pronounced dependence on sulphate groups was observed for adhesion of *H. pullorum*. Bars represent median ± MAD, the *P*-values are for two-tailed tests against heparin.