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Expression of matrix metalloprotease-2, -7, and -9 on human colon, liver and bile duct cell lines by enteric and gastric *Helicobacter* species

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Abstract

Gastric and enteric *Helicobacter* species have been associated with malignant and inflammatory diseases of the stomach, liver, gall bladder and intestine. Matrix metalloproteinases (MMPs) participate in degradation of extracellular matrix (ECM), which allows bacteria to come in contact with and interact with the cells. Enhanced level of MMPs facilitates metastasis and cell invasion of tumor cells by removal of physical barriers, as well as modulation of cell adhesion and biologic activities of proteins residing in the ECM. The aim of this study was to evaluate the effect of gastric and enteric *Helicobacter* on induction of MMPs in hepatocytes and epithelial cells of gall bladder and colon. Human hepatocytes HepG2, gall bladder epithelial cells TFK-1, and colon epithelial cells HT29 were infected with strains of *H. pylori cagA*+, *cagE*+, *H. pylori cagA*-, *cagE*-, *H. pullorum*, *H. cholecystus, H. bilis* and *H. hepaticus*. Protein levels of MMPs were analyzed by enzyme-linked immunosorbent assay and immunohistochemistry. Reverse transcription-quantitative polymerase chain reaction was used to study mRNA levels. Increased expression of MMP-2 and MMP-9 was observed on HepG2, TFK-1 and HT29 infected with *H. pylori cagA*+, *cagE*+ and *H. cholecystus* strains. *H. pylori cagA*+, *cagE*+, *H. cholecystus, H. pullorum*, *H. bilis* and *H. hepaticus* strains increased expression of MMP-7 on HT29, compared to uninfected control cells. The effect of MMP upregulation on HepG2, TFK-1 and HT29 was bacterial dose dependent. *H. pylori cagA*-, *cagE*- strain did not increase expression of MMPs. Inducible MMPs on colon and bile duct epithelial cells as well as hepatocytes cells may play an important role in facilitating invasion and progression of cancer by *Helicobacter* species colonizing the hepatobiliary and gastrointestinal tract.

Keywords: Enteric *Helicobacter*, matrix metalloproteinase, epithelial cells, bile duct, colon, liver

1. Introduction

The *Helicobacter* genus are gram-negative bacteria, and includes more than 20 adequately characterized species to date [1]. These species have been isolated from a number of hosts, such as humans and other primates, pigs, cats, dogs, poultry, and rodents. Moreover, within their hosts, *Helicobacter* species have been identified from both the gastric and the enterohepatic niches of the gastrointestinal tract. *H. pullorum*, first detected in the liver and intestinal contents of poultry [2], has since been isolated from humans with gastroenteritis [3]. *H. bilis* and *H. hepaticus* have been isolated from the liver and the intestinal mucosa from mice, and has been implicated to be associated with rodent chronic hepatitis and liver cancer [4, 5]. *H. cholecystus* has been identified from the gall bladder of hamsters [6]. *H. pylori* is the most widely studied species and is associated with human gastric pathology [7].

The matrix metalloproteinases (MMPs) comprise a family of over 25 members which are secreted from a variety of cells, and are normally found as pro-enzymes in the extracellular matrix (ECM). When these molecules are activated, they participate in degradation of ECM proteins [8], and allows bacteria to come into contact with and interact with the cells. An enhanced level of MMPs also facilitates metastasis and cell invasion of tumour cells by removal of physical barriers, as well as modulation of cell adhesion and biologic activities of proteins residing in the ECM [9]. MMP-9 (gelatinase B) digests denatured collagens, such as the major component of the basement membrane type IV collagen, and has three repeats of a type II fibronectin domain in the catalytic site, which bind to the substrates including collagens, gelatine and laminin [8]. MMP-2 (gelatinase A) is structurally related to MMP-9 and cleaves ECM proteins such as collagen type I and IV [8]. MMP-7 (matrilysin) is the smallest of all the MMPs consisting of a pro-peptide domain and a catalytic domain [8]. It degrades various matrix substrates, including proteoglycans, elastine, and gelatine, and cleaves non-matrix proteins from the cell surface, including E-cadherin, pro-tumour necrosis

factor α and Fas ligand [8]. MMPs can be activated by proteinases, or *in vitro*, by chemical agents, low pH and heat treatment [8].

In two gastric epithelial cell lines, MKN45 and MKN28, MMP-9 expression has been shown to be upregulated by *H. pylori* possessing the cytotoxin-associated gene pathogenicity island (*cag* PAI), though independent of the vacuolating cytotoxin *vac*A locus [10]. The *cag* PAI has also been implicated to associate with upregulation of MMP-7 in colon epithelial cell line HT29 stimulated by *H. pylori* [11]. However, whether the non-gastric enteric species of the *Helicobacter* genus is associated with MMP stimulation has not been investigated, though the bacteria have been linked to inflammatory and ulcerative diseases as well as cancer of the enterohepatic tissue [1]. The **object of this study was to** elucidate the effect on MMP-2, MMP-7 and MMP-9 by enteric and gastric *Helicobacter* species at protein expression as well as gene expression levels.

2. **Materials and methods**

2. 1. Bacterial strains and culture conditions

Five *Helicobacter* strains were obtained from Culture Collection University of Göteborg (CCUG), including four enteric *Helicobacter* strains: *H. pullorum* CCUG 33838, *H. cholecystus* CCUG 38167, *H. bilis* CCUG 38995 and *H. hepaticus* CCUG 33837. *H. pylori* CCUG 17874 *cagA+*, *cagE+*, *vacA s1m1*, and a chronic gastritis isolate *H. pylori* 15/96 *cagA-*, *cagE-*, *vacA s2m2*, **were** typed by PCR. Bacteria were grown on Blood-Brucella agar with 0.1 % activated charcoal in microaerophilic milieu, generated with Anoxomat® (MART Microbiology BV, Licthenvoorde, Netherlands) in vented GasPak jars at 37°C. Bacteria were sub-cultured every second day. For infection experiments, bacteria were harvested from agar plates, re-suspended in warm cell culture media, and were immediately incubated with the cell lines. Gram stain of bacteria was accomplished before sub-cultivation and inoculation with the cells to exclude contamination and to detect the rate of bacterial spiral and coccoid forms.

2. 2. Cell lines and culture conditions

Human cell line cultures were maintained on 75 cm^2 cell culture flasks (TPP AG, Trasadihen, Switzerland). Liver cell line HepG2 and biliary tract epithelial cell line TFK-1 were cultured in RPMI 1640 (ICN, Inc., OH) supplemented with 10 % fetal bovine serum (FBS, Invitrogen, Lidingö, Sweden) and 2 mM L-glutamine (ICN, Inc., OH). Colon epithelial cell line HT29 was grown in McCoy media (Invitrogen, Lidingö, Sweden) with 10 % FBS and 2 mM L-glutamine (ICN, Inc., OH). Cells were incubated at 37°C with 5 % CO_2 and were split once a week. Twenty-four hours prior to infection, cells were transferred to 96-well tissue culture plates (TTP AG, Trasadihen, Switzerland), 100 µl per well, or to chamber glass slides (Nunc Lab-Tek™, Roskilde, Denmark), 2 ml per slide.

2. 3. Enzyme-linked immunosorbent assay (ELISA)

Bacterial doses of 10^5, 10^6, and 10^7 colony forming units (CFU) were inoculated to 2×10^4 cells in order to evaluate the dose dependency of bacterial stimulation for MMP expression. Infected cells on 96-well plates were cultured for 3, 24 or 48 hours at 37°C to evaluate the time course of MMP induction by bacteria. The wells were washed twice with 0.02 M phosphate buffered saline pH 7.2 containing 0.15 M NaCl (PBS), and fixed with 100 % methanol at -20°C for 10 minutes. Endogenous peroxidase activity was blocked with 0.75 % H_2O_2 and 0.1 % NaN_3 in PBS at 37°C for 20 minutes. Cells were then washed, and primary monoclonal antibodies diluted in 1 % bovine serum albumin in PBS were added (MMP-2 and MMP-9, final concentrations 0.2 µg/ml, Santa Cruz Biotechnology, Inc., CA; MMP-7 final concentration 0.3 µg/ml, Oncogene Research Products, Cambridge, MA). The plates were incubated for 1 hour and 30 minutes at 37°C and then washed. Secondary HRP-conjugated antibody (final concentration 0.6µg/ml) was incubated for 1 hour and 15 minutes at 37°C and then washed. Substrate for peroxidase o-phenylenediamine (Sigma-Aldrich, St. Louis, MO) was incubated for approximately 15 minutes in dark, and the reaction was stopped by addition of 1 M H_2SO_4. The absorbance was measured on a spectrophotometer (Labsystem Multiscan PLUS, Labsystem OY, Helsinki, Finland) at 492 nm.

2. 4. Immunohistochemistry

Helicobacter strains 2×10^7 CFU was inoculated to 4×10^5 cells of HT29, HepG2 or TFK-1. After 24 hours of co-cultivation, the chamber slides were washed and the cells were fixed with acetone at -20°C for 10 minutes. Slides were then washed in PBS and endogenous peroxidase activity was blocked by addition of PBS supplemented with 0.03 % H_2O_2 and 0.1 % NaN_3. After washing, primary monoclonal antibodies **against** MMP-7 (final concentration 3 µg/ml, Oncogene Research Products, Cambridge, MA), MMP-2 or MMP-9 (final

concentrations 2 µg/ml, Santa Cruz Biotechnology, Inc., CA) were added and incubated for 2 hours at room temperature. **Normal mouse serum (final concentration 20 µg/ml, DakoCytomation, Älvsjö, Sweden) was used as isotype control.** Cells were washed and incubated with horse radish peroxidase (HRP)-conjugated secondary anti-mouse antibody (final concentration 20 µg/ml, DakoCytomation, Älvsjö, Sweden) for 30 minutes at room temperature. The substrate for peroxidase 0.05 % 3,3'-diaminobenzidine-tetrahydrochloride (Sigma-Aldrich, St. Louis, MO) in PBS was added for 2 minutes and the reaction was stopped with H_2O. Counter staining of the nuclei was performed by hematoxylin for 8-10 seconds, stopped with H_2O. The slides were dehydrated in 70 % ethanol, 95 % ethanol, 99.5 % ethanol and finally in xylene, and mounted with DePeX (Sigma-Aldrich, St. Louis, MO). The slides were examined on an inverted microscope (Olympus CK40, Tokyo, Japan).

2. 5. RNA isolation, primer design, and reverse transcription-quantitative polymerase chain reaction (RT-QPCR)

Total RNA was extracted from 2 × 10^6 cells of HT29, HepG2 and TFK-1, infected with *Helicobacter* strains 1 × 10^8 CFU, or uninfected, by using RNeasy Mini kit with QIA shredders (Qiagene GmbH, Hilden, Germany). Quantity and purity of RNA were measured by A_{260}/A_{280} adsorption using U-110 spectrophotometer (Hitachi, Tokyo, Japan). The different primer pairs used in this study were constructed using Vector NTI® Suite version 8.0 (InforMax, Frederick, Maryland, USA). All primers used in this study were purchased from MWG-Biotech AG, Ebersberg, Germany; MMP-7 forward: 5'-TTTGATGGGCCAGGAAACAC-3', MMP-7 reverse: 5'-GGGGATCTCCATTTCCATAG-3', MMP-9 forward: 5'-GAAGATGCTGCTGTTCAGCG-3', MMP-9 reverse: 5'-ACTTGGTCCACCTGGTTCAA-3', glyceraldehydes-3-phosphate dehydrogenase (GAPDH) forward: 5'- CTCATTTCCTGGTATGACAACGAA-3', GAPDH reverse 5'-

AAGGGGTCTACATGG CAACT-3'. To reduce the effect of DNA contamination in the extracted RNA, the forward primers were designed to cross intron/exon boundaries to ensure that the products detected resulted from amplification of cDNA rather than genomic DNA. The primer specificity was determined by using BLASTN 2.2.1 (http://www.ncbi.nlm.nih.gov/BLAST/) [12]. For RT-QPCR, total RNA (0.5 µg) was reverse transcribed in 20 µl reaction mixture containing 1 × reaction buffer, 0.5 mM deoxynucleoside triphosphates (dNTPs), 10 mM dithiothreitol, RNase inhibitor 40 units (Invitrogen, Lidingö, Sweden), oligo(dT) primer 2 µg (Amersham Plc., Little Chalfont, Buckinghamshire, UK), and Superscript II reverse transcriptase 200 units (Invitrogen, Lidingö, Sweden) at 42°C for 50 minutes. The cDNA (2 µl) was amplified in a 25 µl reaction mixture containing 0.2 mM of each dNTPs, 0.5 µM of forward and reverse primers, 1/100,000 Sybr Green I (Roche Diagnostics Scandinavia, Bromma, Sweden), **1 U Ampli*Taq* DNA polymerase and 1 × PCR buffer (Applied Biosystems, Foster City, CA, USA).** Amplification and detection of amplified PCR products was done by using the Rotorgene (Corbett Research, Sydney, Australia). Amplification conditions were predenaturation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 20 seconds, annealing at 57°C (MMP-7) or 53°C (MMP-9 and GAPDH) for 20 seconds, and extension at 72°C for 20 seconds. The specificity of the amplification was determined by melting curve analysis and gel electrophoresis analysis of the amplified PCR product. Melting curve analysis of the amplified PCR products was done immediately after PCR, by increasing the temperature linearly from 60 to 95°C at a rate of 1°C per second with the signal acquisition mode set to continuous. The threshold cycle (C_T), which is the intersection between each fluorescence curve and the crossing line, was calculated using the Rotorgene software. Simultaneously, 10 µl of PCR products were analysed by electrophoresis in a 1.5 % agarose gel containing ethidium bromide (final concentration 0.5 µg/ml). PCR product size was estimated by Marker Plus (Fermentas GmbH,

St. Leon-Rot, Germany), which was run in parallel. The expression of target genes was normalized against GAPDH gene in a corresponding sample. MMP-7 gene expression in *Helicobacter*-infected cells was presented as fold induction compared to the uninfected control cells: Fold induction $_{\text{MMP-7}}$ = (GAPDH $C_{T\ \text{infected}}$ / MMP-7 $C_{T\ \text{infected}}$) / (GAPDH $C_{T\ \text{uninfected}}$ / MMP-7 $C_{T\ \text{uninfected}}$). Equivalent formula was utilized to calculate fold-induction of MMP-9 genes.

2. 6. Statistics

Mann-Whitney *U* test was performed to analyze the significance of MMP induction in human cell lines infected by *Helicobacter* strains compared to uninfected control cells. A *p* value of <0.05 was considered as significant difference.

3. Results

3.1. Optimization of conditions for induction of MMP-2, MMP-7 and MMP-9 expression on HT29, HepG2 and TFK-1

MMP-2, MMP-9 and MMP-7 on HT29, HepG2 and TFK-1 was measured by ELISA subsequent to 3, 6 and 24 hours of infection with *H. pylori* 17874, which showed an increase of expression by time reaching maximum levels at 24 hours (data not shown). In a separate experiment, 10^5, 10^6 and 10^7 CFU of *H. pylori* 17874 were infected with 2×10^4 cells for 24 hours, which resulted in significant upregulation of MMP expression compared to uninfected control cells when more than 10^6 CFU was added, and the MMP expression levels increased in a bacterial dose dependent manner (data not shown). Therefore, each of the six *Helicobacter* strains was inoculated with cells at a ratio of **50:1** for 24 hours to analyze MMP induction on enterohepatic cell lines by ELISA, immunohistochemistry and RT-QPCR.

3.2. ELISA of MMP-2, MMP-7 and MMP-9 on HT29, HepG2 and TFK-1

ELISA revealed significant increase of MMP-2 and MMP-9 expression on HT29, HepG2 and TFK-1 infected by *H. pylori* 17874 and ***H. cholecystus***, compared to uninfected control cells, while the other enteric *Helicobacter* had only minor effects on upregulation of MMPs on these cell lines (Fig1, 2). When expression of MMP-7 was analyzed on HT29, *H. pylori* 17874, ***H. pullorum, H. cholecystus, H. bilis* and *H. hepaticus*** significantly increased expression, whereas the effect of *H. pylori* 15/96 on expression of MMP-7 was not significant (Fig 3).

3.3. Immunohistochemistry of MMP-2, MMP-7 and MMP-9 on HT29, HepG2 and TFK-1

HT29, HepG2 and TFK-1 infected with *H. pylori* 17874 and ***H. cholecystus*** showed specific positive reaction with antibodies against MMP-2 and MMP-9, whereas uninfected control cells did not (data not shown). HT29, HepG2 and TFK-1 infected with ***H. pullorum,*** *H. pylori* 17874, ***H. cholecystus, H. bilis* and *H. hepaticus*** showed specific positive reaction for MMP-7, but not those infected with *H. pylori* 15/96 or the uninfected control (Fig 4 **and data not shown**).

3.4. RT-QPCR of ~~MMP-2,~~ MMP-7 and MMP-9 in HT29, HepG2 and TFK-1

RT-QPCR analysis revealed **higher levels** of MMP-7 mRNA in HT29 by **3 hours of infection with *H. pullorum***, *H. pylori* 17874 and ***H. cholecystus,*** but not by *H. pylori* 15/96 or those uninfected (Fig 5). MMP-7 mRNA in HT29 **induced by *H. pullorum***, *H. pylori* 17874 and ***H. cholecystus,*** remained at **higher levels** than those uninfected **at 6 and 24 hours of infection (data not shown)**. TFK-1, HepG2 and HT29 infected with *H. pylori* 17874 and ***H. cholecystus*** demonstrated **higher levels** of MMP-9 mRNA **at 3, 6 and 24 hours of infection**, whereas those infected with *H. pylori* 15/96 or uninfected did not (data not shown), consistent with the results of ELISA and immunohistochemistry.

4. Discussion

The present study shows that the oncogenic pathogen *H. pylori* infection causes upregulation of MMP-2, MMP-7 and MMP-9 in human enterohepatic cells, and that it is dependent on intact *cag* PAI. Moreover, we have demonstrated that enteric species of the *Helicobacter* genus are also capable in upregulation of MMP-2, MMP-7 and MMP-9 in colon and bile duct epithelial cells and hepatocytes. In contrast to other inflammatory mediators such as the chemokine IL-8, which has been shown to be upregulated within few hours after *H. pylori* infection [13], expression of MMP-2, MMP-7 and MMP-9 by *Helicobacter* species was prolonged to a later phase of infection~~, which may indicate a potent role of MMPs in *Helicobacter* infection in progression to advanced stages of diseases of the gastroenteric and hepatobiliary tracts~~. MMP-2, MMP-7 and MMP-9 are important metalloproteinase enzymes which are **upregulated** in enterohepatic cancers and have been considered to be important markers in human cancer progression. Links between MMP-9 in bile with liver metastasis in colorectal cancer [14] and MMP-2 in advanced cholangiocarcinoma in contrast to normal or non-metastatic livers [15] have been reported. In animal models, *Min* (multiple intestinal neoplasia) mice developed adenomas in the small intestine and colon, whereas MMP-7-deficient *Min* mice resulted in fewer tumors [16]. MMPs promote tissue invasion and metastasis in various cancers through degradation of ECM. The type IV collagenases MMP-2 and MMP-9 have been found to play a key role in the invasive ability of tumors, as basement membrane is composed primarily of type IV collagen [8]. In addition, substrates of MMP-7 include pro-apoptotic Fas ligand. Thus, decreasing the ability to suppress apoptosis may predispose to proliferation and tumorgenesis [17].

In the present study, expression of *Helicobacter*-induced MMPs was bacterial dose dependent, indicating that stimulation of enterohepatic cells in induction of MMPs may

require a specific ligand-receptor interaction. Exposure of mouse or human mucosal epithelial cells to other gram-negative bacteria, such as *Escherichia coli* and *Pseudomonas aeruginosa*, induced a strong and sustained induction of MMP-7 by the monomeric component of flagella, flagellin, but not by lipopolysaccharide [18]. The *cag* PAI of *H. pylori* encodes components of the type IV secretion system by which the bacteria attach to the cells and delivers the CagA protein into the cytoplasm [13]. Crawford *et al.* has reported in gastric epithelial cell line AGS that *H. pylori* possessing the *cag* PAI, but not the *cag* mutants, induced expression of MMP-7 through intracellular signal pathway involving extracellular signal regulated kinase 1/2 activation [19]. Also in AGS, Wroblewski *et al.* reported that MMP-7 induction by *cag* positive *H. pylori* involved Rac activation through nuclear factor kappa B (NF-κB), and Rho activation through NF-κB and activator protein 1 [20]. The involvement of *cag* PAI seems crucial in MMP expression, since in our study, the *H. pylori cagA*+, *cagE*+ strain induced expression of MMP-2, MMP-7 and MMP-9 in bile duct and colon epithelial cells, as well as hepatocytes, whereas the *H. pylori cagA*-, *cagE*- strain did not. However, *cag* PAI may not be the only pathogenic factor to induce MMPs, since the enteric *Helicobacter*, which lack *cag* PAI, were demonstrated in the present study to induce significant levels of MMP-7 on colon epithelial cells. Moreover, *H. cholecystus* stimulated MMP-2 and MMP-9 expression on colon and biliary tract epithelial cells as well as hepatocytes, as did *cag* positive *H. pylori*. **Although all the enteric bacteria investigated in the present study are members of the genus *Helicobacter*, they are different species associated with enterohepatic diseases in various hosts, most of which have not been studied in the same depth to date as *H. pylori* regarding virulence factors.** In other enteric bacteria that share the enteric niches for colonization, cytolethal distending toxin (CDT) has been demonstrated to play an important role in pathogenicity, as has been demonstrated in several enteric *Helicobacter* strains [21]. All four strains of enteric *Helicobacter* used in the present study possess *cdtB*, which encodes

the functional domain of CDT, confirmed by PCR by using primers generated from species specific *cdtB* sequence (Abu Al-Soud, unpublished data). However, heterogenic expression of MMPs induced by the enteric *Helicobacter* strains, may suggest an involvement of pathogenic factors other than CDT. Further investigation on the pathogenic factors of enteric *Helicobacter* responsible for MMP induction is needed.

Considerable evidence to support the role for MMPs in progression of inflammatory and oncogenic diseases of the hepatobiliary tract as well as the gastrointestine [22] may encourage further strategies for treatment and prevention of the diseases to include use of inhibitors of metalloprotease enzymes [17]. Stimulation of MMP expression on enteric and biliary epithelial cells as well as hepatocytes may potentially be an important pathogenic effect of the *Helicobacter* species colonizing the hepatobiliary and the gastrointestinal tract.

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Figure legends

Figure 1. Effect of gastric and enteric *Helicobacter* strains on MMP-2 expression. ***H. pullorum,*** *H. pylori*17874, *H. pylori* 15/96, ***H. cholecystus, H. bilis* and *H. hepaticus* strains described in materials and methods** were co-cultured with HT29, HepG2 or TFK-1. The expression level of MMP-2 was measured at 24 hours, and was compared to the uninfected cells. Optical density of wells incubated with secondary antibody only was subtracted from the sample data. Data presented are mean values (error bars, standard deviation) of **nine samples from three separate experiments performed in triplicates**. **p*<0.05, ***p*<0.01, ~~****p*<0.001,~~ Mann-Whitney *U* test.

Figure 2. Effect of gastric and enteric *Helicobacter* strains on MMP-9 expression. ***H. pullorum,*** *H. pylori*17874, *H. pylori* 15/96, ***H. cholecystus, H. bilis* and *H. hepaticus* strains described in materials and methods** were co-cultured with HT29, HepG2 or TFK-1. The expression level of MMP-9 was measured at 24 hours, and was compared to the uninfected cells. Optical density of wells incubated with secondary antibody only was subtracted from the sample data. Data presented are mean values (error bars, standard deviation) of **nine samples from three separate experiments performed in triplicates**. **p*<0.05, ~~***p*<0.01,~~ Mann-Whitney *U* test.

Figure 3. Effect of gastric and enteric *Helicobacter* strains on MMP-7 expression. ***H. pullorum,*** *H. pylori*17874, *H. pylori* 15/96, ***H. cholecystus, H. bilis* and *H. hepaticus* strains described in materials and methods** were co-cultured with HT29. The expression level of MMP-7 was measured at 24 hours, and was compared to the uninfected cells. Optical density of wells incubated with secondary antibody only was subtracted from the sample data. Data presented are mean values (error bars, standard deviation) of **nine samples from three separate experiments performed in triplicates**. **p*<0.05, Mann-Whitney *U* test.

Figure 4. Immunohistochemistry of HT29 infected with gastric and enteric *Helicobacter* strains. HT29 cells were co-cultured with ***H. pullorum*** (B), *H. pylori* 17874 (C), *H. pylori* 15/96 (D), ***H. cholecystus*** (E), ***H. bilis*** (F) **and *H. hepaticus*** (G) **strains described in materials and methods** for 24 hours, or left uninfected (A). Antibody against MMP-7 was visualized by DAB substrate, counter stained with hematoxylin. Original magnification ×10.

Figure 5. RT-QPCR of HT29 for MMP-7. Cells were co-cultured with ***H. pullorum***, *H. pylori* 17874, *H. pylori* 15/96 and ***H. cholecystus* strains described in materials and methods** for 24 hours, or left uninfected. Total RNA was reverse transcribed and cDNA was amplified in a Real-Time DNA thermal cycler. Threshold cycle (C_T) was calculated on Rotorgene software (Corbett Research). Data are fold induction of Ct^{-1} values normalized against GAPDH mRNA in a corresponding sample. **Data presented are mean values of triplicated samples in one representative experiment of three performed.**

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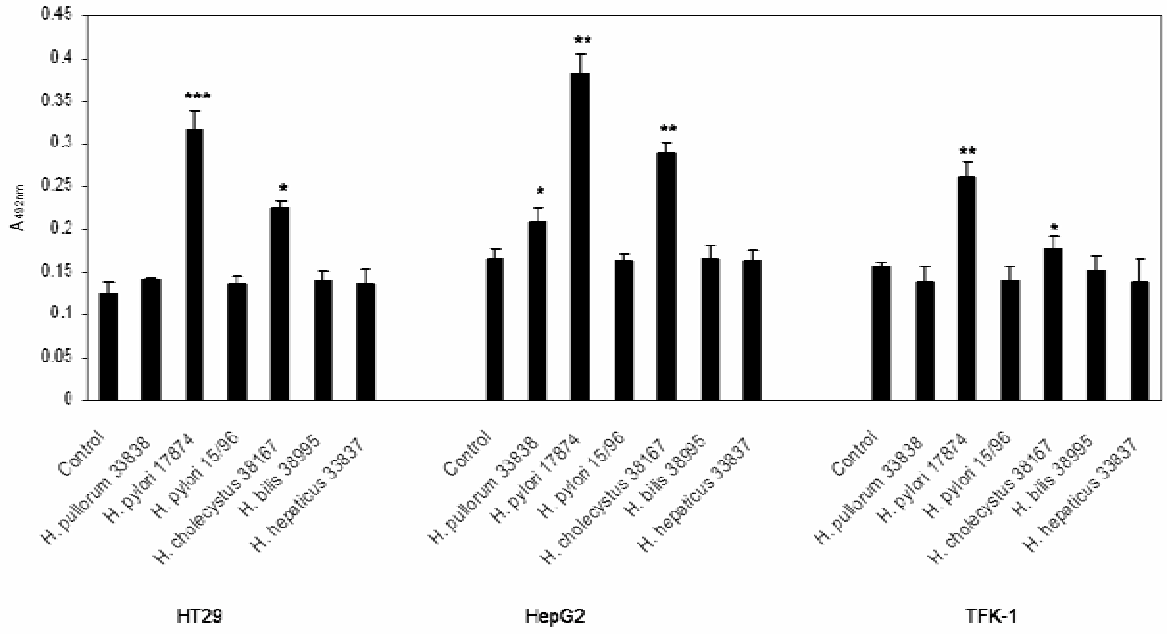
Figure 1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H. hepaticus 33837
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HepG2
TFK-1

Figure 2

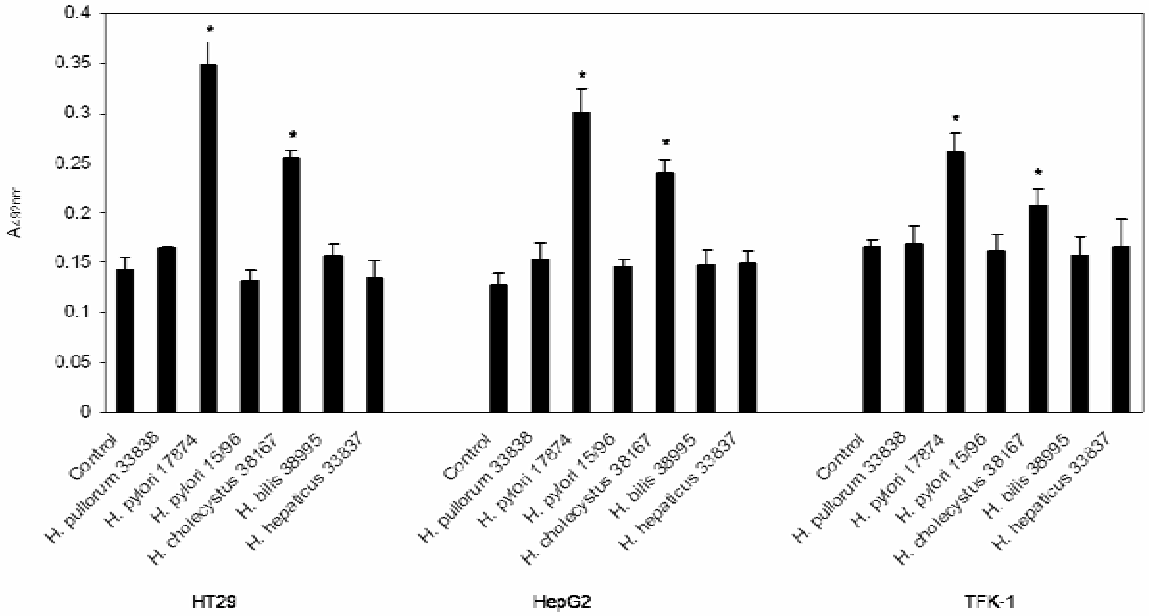
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Figure 3

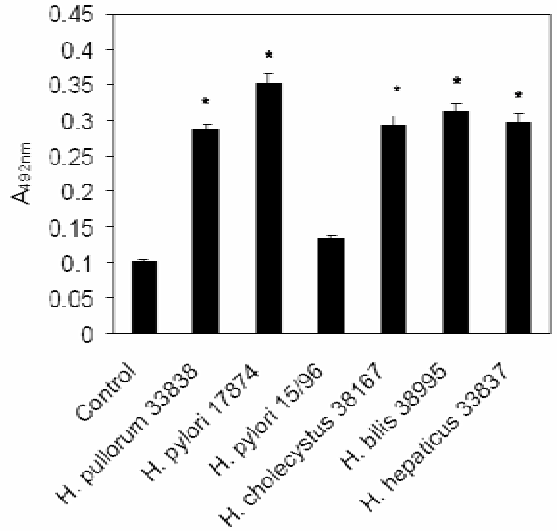
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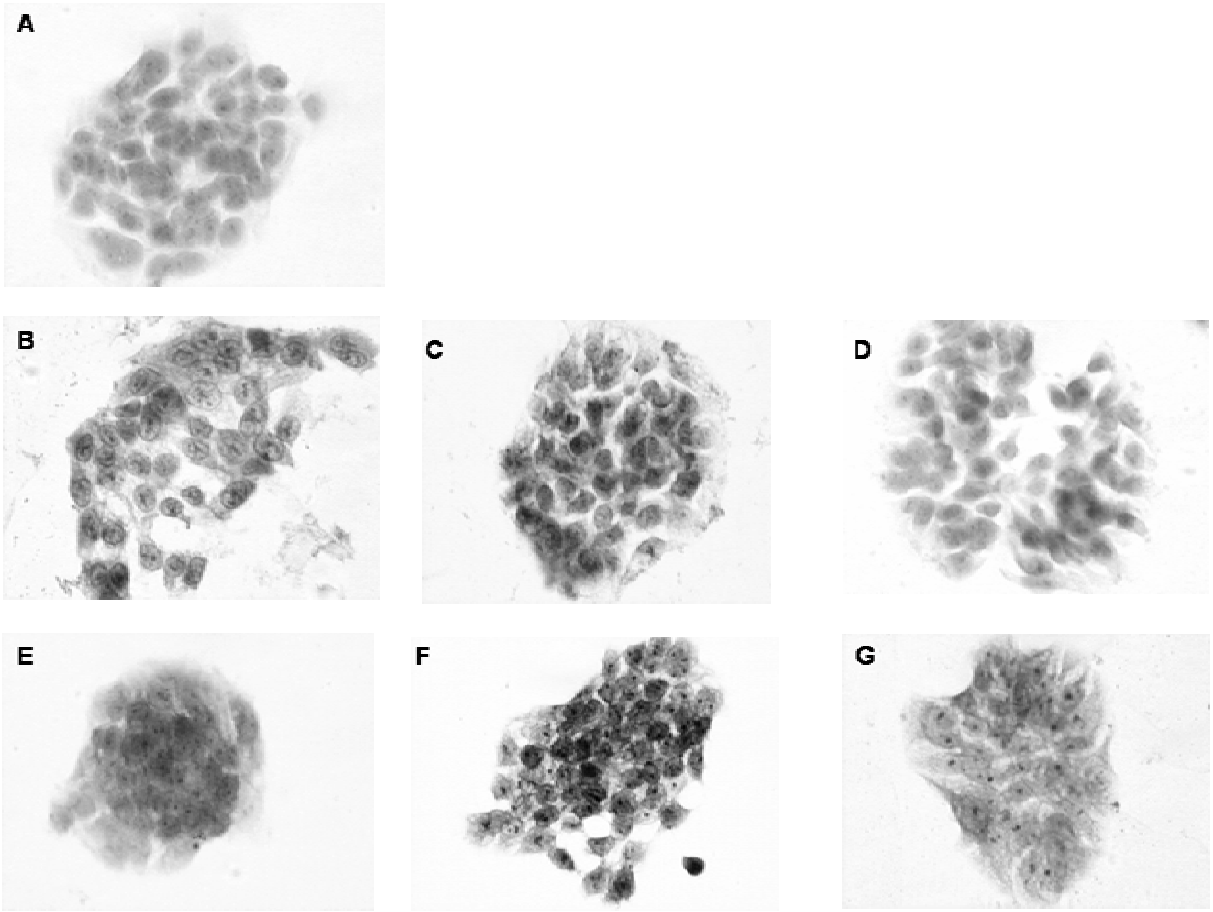
Figure 4



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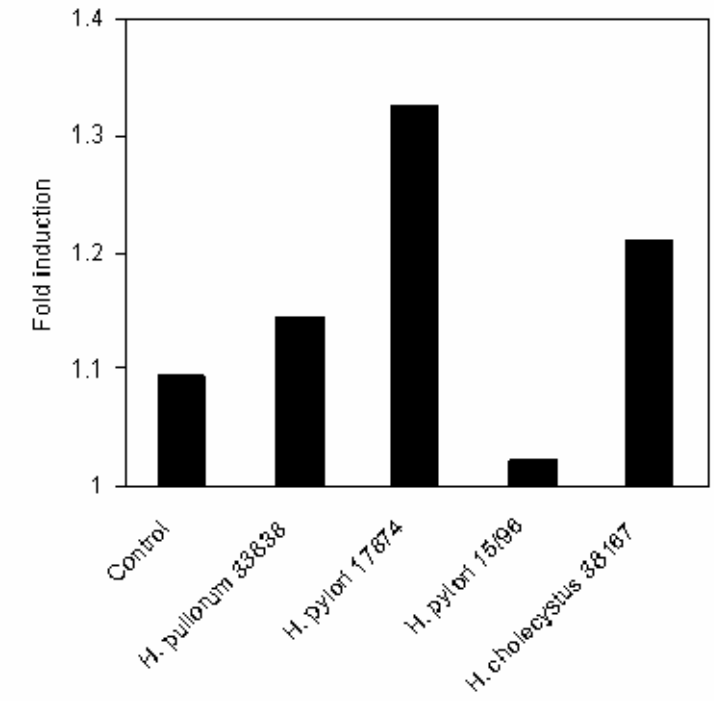
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