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Virulence Factors in Non-Gastric *Helicobacters*

by

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Abstract

Helicobacter bilis is one of several *Helicobacter* species that is associated with disease. *F. rappini* is very similar in all respects to *H. bilis* except that it has not been documented to colonize the liver of mice, nor has it been associated with liver disease. If in fact *F. rappini* can not cause liver disease, then differences in these genomes are likely to be involved in the colonization in the liver and/or pathogenesis of liver disease by *H. bilis*. The two species were characterized to identify phenotypic differences, which might reflect the differences in pathogenicity.

Two phenotypic factors expected to be involved in pathogenesis are survival in the presence of bile salts in the liver and attachment to liver cells. Bile assays were designed and performed to study the survival of the different *Helicobacter* strains in the presence of bile salts. Results showed that there is a difference between the species, and using optimized conditions a clear pattern of resistance can be seen in *H. bilis*. Adherence to tissue culture cells was also characterized to see if there were phenotypic differences between the strains. Results from the adherence assays show a significant increase in the adherence of *H. bilis* over the *F. rappini* strain.

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Introduction:

Helicobacters have been known for over 15 years to be involved with chronic inflammatory diseases. *H. Pylori*, the most common gastric *Helicobacter* in humans, is known to be involved with chronic gastritis and peptic ulcer disease (Blaser 1992; Lee, Fox et al. 1993). The gastritis is associated with chronic inflammation with mononuclear and neutrophil infiltration leading to peptic ulceration. There is an increase in cell proliferation as well as glandular atrophy. It has also been associated with gastric adenocarcinoma and gastric mucosa associated (MALT) lymphoma and recently the International Agency for Research on Cancer has also named it a definite carcinogen (Parsonnet 1995).

Two other gastric *Helicobacters* that cause disease in their hosts are *H. mustelae* and *H. felis* (Fox, Correa et al. 1990; Fox, Otto et al. 1991; Fox, Blanco et al. 1993). *H. mustelae* causes chronic inflammation in the stomachs of naturally infected ferrets. In mice infected with *H. felis*, a mostly mononuclear leukocyte infiltrate in the stomach as well as some polymorphonuclear involvement is seen. These are used as animal models of chronic inflammation because of their similarity to *H. pylori* infections.

Recently, attention has been brought to the non-gastric *Helicobacter* species, *H. muridarum*, *H. hepaticus*, *H. bilis*, and *H. rappini*. All of these species have been associated with various disease states in their hosts as seen with the gastric species. *H. hepaticus* causes chronic active hepatitis in mice and has also been associated with liver tumors in A/JCR mice (Fox, Li et al. 1996; Fox, Yan et al. 1996). *H. pullorum* has been isolated from adults and children with gastroenteritis. (Burnens, Stanley et al. 1994; Stanley, Linton et al. 1994). *H. muridarum* and *F. rappini* have been associated with gastritis in older mice and abortion in sheep, respectively (Lee, Phillips et al. 1992).

H. bilis and *F. rappini*, an officially unnamed *Helicobacter*, are the most closely related of the murine *Helicobacters*. *F. rappini* is 98% similar to *H. bilis* as judged by 16s RNA sequence data. Both of these have similar physical and biochemical properties (Schauer, Ghori et al. 1993; Fox, Yan et al. 1995). They are both spiral shaped, measure 0.5 by 4-5 um, have sheathed flagella, periplasmic fibers. Growth at 42°C, and have oxidase,

urease, and catalase activity. *F. rappini* is a morphologically defined group that consists of multiple species. Some species are associated with gastroenteritis in humans, or abortion in sheep, but are not associated with disease in mice or hepatitis. Because *F. rappini* consists of multiple species, there may be multiple disease states associated with it. The main difference between these two organisms is that *H. bilis* inhabits the liver and is associated with chronic hepatitis *and F. Rappini* is not.

H. bilis is associated with hepatitis in aged mice (Fox, Yan et al. 1995). *H. bilis* is one of two *Helicobacters* known to colonize the liver of mice. The bacteria may be more widespread than is currently known and may be able to cross species barriers. Recently *H. bilis* was isolated from a dog with gastritis (Eaton, Dewhirst et al. 1996). If it is possible for *H. bilis* to cross species barriers, then it may be possible that *H. bilis* as well as other *Helicobacters* may be found to be the cause of some cases of idiopathic hepatitis in humans. It is important to study the factors involved in liver disease by *H. bilis* because it may give insight into causes of bacterial hepatitis in rodent species as well as other mammalian species. There is not much known about how *H. bilis* colonizes the liver or is involved in the progression of hepatitis in mice. *H. bilis* may provide a mouse model to study this process.

Even though these species all cause varying diseases and colonize different hosts, the common factor may be in the mechanism of pathogenesis. In all the cases of gastroenteritis mentioned above, there is a chronic inflammatory response to the infections. It is this chronic inflammatory response that is believed to be responsible for the increased risk of gastric carcinoma in the case of *H. pylori*. There are many common virulence factors associated with theses species that may be involved in the inflammatory process. Therefore studying the pathogenesis of one species may lead to helpful insights that will be useful in other models of bacterial causes of chronic inflammation

Several virulence factors have been found in *H. pylori* that enable the bacteria to colonize the stomach and cause disease. The flagella permit *H. pylori* to remain motile in the gastric mucus layer and aid in colonization. Both flagella genes are required for persistent infections in the gnotobiotic piglet model (Eaton and Krakowka 1992) (Eaton, Suerbaum et al. 1996). Urease may also aid in colonization by neutralizing the gastric acid

by the production of ammonia. Urease negative *H. pylori* are reduced in their ability to colonize germfree piglets (Eaton and Krakowka 1994). The *cag* (cytotoxin-associated gene) pathogenicity Island has been associated with induction of inflammation (Christie 1997; Covacci, Falkow et al. 1997). It is the neutrophil response that is believed to cause damage to the mucosal surface and gastric cells. Many of the other *Helicobacter* species have these same virulence factors.

One virulence factor, the ability to have resistance to bile salts, varies among the *Helicobacters*. Bile salts are detergents produced by the liver that kill most gram-negative bacteria. Cholic and chenodeoxycholic acids are primary bile acids that are produced in the liver. The secondary bile acids, deoxycholic and lithocholic acids, are produced in the gut from the primary bile acids. It is obvious in the case of *H. bilis* that the ability to survive in the presence of bile is important for colonization in the liver. Previous reports have shown that gastric *Helicobacters* like *H. pylori* are sensitive to 1% ox gal bile and intestinal *Helicobacters* like *F. rappini* are resistant to 1% ox gal bile (Fox, Yan et al. 1995). *H. bilis* is unusual in that it is highly resistant to ox gal bile as high as 20%. Because ox gall bile is a crude sample of bile and there is no batch to batch consistency, assays were designed to quantitate the response of *Helicobacters* to individual bile salts. Looking at individual bile salts, it was found that *H. pylori* is inhibited by 0.1% chenodeoxycholic acid and 40% of the strains tested were inhibited by ursodeoxycholic acid. Deoxycholic acid (DCA) has been shown to be the most toxic of the bile salts, inhibiting growth tested as low as 0.5mM in *H. pylori* (Hanninen 1991; Mathai, Arora et al. 1991). This is likely to be a factor in the survival of *H. bilis* in the liver of mice. The conditions for the bile assays were tested here to show the phenotypes of the different *Helicobacters* in the presence of DCA.

Another virulence factor that may be important in *H. bilis* colonization is the adherence of the bacteria to tissue culture cells. This is important to characterize because adherence to cells is a virulence factor in many other bacterial systems and may be just as important in this case. There are two ways in which adherence can act as a virulence factor: the bacteria may be binding to a certain cell type or the binding may trigger signaling events within the host cell. The adherence of *H. pylori* to gastric cells is believed

to induce signaling events (Segal, Lange et al. 1997). With all the similarities in the *Helicobacter* species, it is very possible that a comparable event is involved with *H. bilis*. Adherence assays were performed on *H. bilis* and *F. rappini* to see if different adherence patterns exist that could explain differences in their pathogenicities.

Methods:

Bacteria and growth conditions:

H. bilis 1909 (ATCC 51630), *F. rappini*, and *H. pylori* (type strain, ATCC 43504) were continually grown on tryptic soy agar (Sigma, St. Louis, MO), (2.5 % agar, 5% sheep blood (Remel, Lenexa, KS)) plates for two days in a sealed gaspack jar at 37°C under microaerophilic conditions. For all assays the bacteria were scraped off the plates and resuspended in tryptic soy broth to an OD_{600} 1.0 to standardize the starting concentrations for the assays.

Bile assay:

The standardized bacteria were used to inoculate 5 mls of tryptic soy broth (5% FBS) in a T-25 tissue culture flask (Corning). A dilution ranging between 1:50 and 1:300 was used as the inocculum. Deoxycholic Acid (Sigma, St. Louis, MO) was then added to a final concentration between 0 mM and 0.5 mM. The flasks were placed on their side and sealed in a microaerophilic atmosphere and incubated at 37°C for 48 hrs. The jars were gently shaken at 40 rpm. The OD_{600} was checked at time 0, 12, 36, and 48 hours. To check the OD_{600}, 1 ml was taken from each of the samples. The OD readings from the 0-hr time point were used to determine the % inhibition for the samples. The % inhibition was calculated as follows:

$$\frac{OD_{600} \text{ at tested DCA conc.} - OD_{600} \text{ at 0mM DCA}}{OD_{600} \text{ at 0mM DCA}}$$

Adherence Assay:

Tissue culture cells (HEP-2 (ATCC CCL-23) and BNLC.2(ATCC TIB-73) were grown in DMEM, 10% FBS in 5% CO_2 until confluent. The night before the assay, cover

slips were seeded with 2 x 10^5 tissue culture cells in a 24 well plate. On the day of the assay the bacteria were resuspended in tryptic soy broth as described above. The tissue culture cells were inoculated with 50 ul of the bacterial suspension and spun for 10 minutes at 2000 rpm. The samples were incubated in 5% CO_2 incubator for 6 hours. The cover slips were washed 6 times with sterile PBS, then stained for 1 minute each in fixative, stain 1 and stain 2 from DifQuik stain. They were washed 3 times with H_2O, and then let to dry overnight and the coverslips were mounted on slides the next day. To quantitate the adherence, 50 cells were counted in a straight line, with the starting cell selected at random. The number of bacteria associated with those 50 cells was counted in triplicate, and each sample was done in triplicate.

Results:

Bile Resistance:

It was necessary to standardize the assay, in order to get consistent results from the bile assays. Two factors needed to be determined: the starting concentration of bacteria and the concentration of DCA. The *H. bilis* growth curve was used to determine the staring concentration of the bacteria. When the bacteria aren't diluted enough, they loose the lag phase of the growth curve and might miss the effect of killing vs. just stopping the growth. If they are diluted too much, they won't grow at all. The conditions used in the figure 1 give consistent growth patterns. Using these conditions in the bile assays would give reproducible results. Using this curve, it was determined that the desired starting dilution for these assays would be 1:50 of an OD_{600} 1.0. This gives an ideal growth curve with all three phases, lag, exponential, and stationary.

The next assays to be done were to determine the concentration of Deoxycholic acid that would show a difference between these two species of bacteria. These assays were only tested for their final growth concentrations, not growth curves. In figure 2, a range between 0 and 0.5 mM was tested. *F. rappini* is inhibited 70% at 0.25 mM DCA, while *H. bilis* is only 12% inhibited. However, at higher concentrations of DCA (0.5 mM) there is no difference at all. These were done at a 1:100 dilution to give a maximum growth, which may lead to elevated growth levels. This clearly shows the phenotypic difference between these species at a level of DCA that is lethal to *H. pylori*.

In a bile assay with just *H. bilis*, the growth curve was observed in the presence of DCA. Without the addition of DCA, the growth curve is the same as previously shown in figure 1. In 0.25 mM DCA *H. bilis* is inhibited 31% and in 0.5 mM it is inhibited 68% (figure 3). These are levels that have been previously shown to be lethal to *H. pylori*. One observation was that the shape of the growth curve was changed in the presence of DCA. The lag phase was extended as the level of DCA increases.

Adherence assay:

These assays show that a difference in the adherence of these bacteria does exist. Adherence was first looked at in Hep – 2 cells, a human epithelial cell line. A significant difference was seen in the adherence pattern between *H. bilis* and *F. rappini* (T test, $p < .001$) (Figure 4). *H. bilis* had 70 bac/50 cells, while *F. rappini* had 30 bac/50 cells. There was no difference between a strain of *H. bilis* (65 bac/50 cells) passaged through a mouse for 6 months and a lab passaged strain. The other cell line tested gave similar results. In figure 5, a significant difference can be seen between these two strains in their adherence to BNLC.2, a liver cell line. *H. bilis* had 45 bac/50 cells and *F. rappini* had only 15 bac/50 cells (T test, $p < .001$).

Discussion:

Clearly there are phenotypic differences between these two closely related species of *Helicobacter*. This could be due to various factors. One possibility is that *F. rappini* has had a loss of function mutation occur in a virulence factor that does not allow *F. rappini* to colonize the liver. This could occur in a number of ways. One would be a change in a regulatory factor. This could be a large or small deletion or a single base pair change that makes a nonfunctional regulatory protein. Another possibility is that *H. bilis* contains a pathogenicity island (PAI) that *F. rappini* does not. This is a relatively new term describing a group of virulence genes in bacterial chromosomes that is a distinct functional unit. It is possible that *H. bilis* contains a PAI and it has been either lost in *F. rappini* or was never inserted. A similar loss can been seen in *Yersinia pestis*. A spontaneous deletion of 102 kb results in the loss of virulence of *Y. pestis* (Lee 1996). PAI's have even been found in *H. pylori*. There is a CAG (cytotoxin associated gene) pathogenicity island that contains many virulence factors in *H. bilis* (Christie 1997; Covacci, Falkow et al. 1997).

Either way the genotypic differences arose in theses species the end result is the same. *H. bilis* is able to colonize the liver and cause disease and *F. rappini* does not. Studying the differences between these strains can help to understand the bacterial etiology of hepatitis and possibly the mechanism of inflammation seen with other *Helicobacter* associated diseases.

Helicobacter strains were tested in vitro for resistance to bile salts because the bacteria must be able to survive in the liver if they are to colonize and cause hepatitis. The results of the bile assays show that it is possible to get consistent, reproducible results from a standardized bile assay. By testing every twelve hours, it was possible to detect an extended lag phase in the presence of DCA. The cause of the lag is unknown, but there are three possibilities to explain this observation. The first is that the lag is due to the breakdown of the DCA after 24 hrs. This could easily be tested by letting an un-innoculated sample sit for 24 hours before adding the bacteria. If the lag phase disappears, then the response was due to the DCA breakdown. If however the lag phase is still

present, then the response is due to the bacteria. The second possibility is that the assay is selecting for a mutant population that is resistant to the DCA. To test this possibility the outgrowth from the assay should be tested again in the same assay. If the lag phase is still seen, then the response would be the third possibility, a slower growth in the presence of DCA. If the lag disappears, then the response would be sue to the selection of a mutant population.

The preliminary results from the *F. rappini* assay confirm what was already seen in the literature. At levels of DCA that were lethal to *H. pylori* (0.25 mM), *F. rappini* were able to survive. However, at even higher levels of DCA (0>5 mM), *F. rappini* was inhibited, but *H. bilis* was still able to grow. This may be why *F. rappini* has not been found to colonize the livers of animals. The bile salts are produced there and are therefore in their greatest concentration in the liver. *H. bilis* may be one of the few bacteria able to survive in such high levels of bile acids allowing colonization to occur.

Now that there is evidence that this assay is consistent and reproducible, in can be used in many ways. First other *Helicobacters* can be tested for their response to DCA. Second, other bile acids can be used to see what difference exists between primary and secondary bile acids. It will also be useful to see if there might be an adapted response to living in the mouse gut. Maybe bacteria are more resistant after being passaged though the mouse. When looking at mutations, this assay can be used to isolate mutants that have lost this response to find the responsible genes.

Adherence to cell lines is an important virulence factor in many bacterial systems. These assays were designed to look at phenotypic differences in adherence patterns. In both cell lines tested, *H. bilis* was more adherent than *F. rappini*, but there was no difference seen with a mouse passaged strain of *H. bilis*. What hasn't been determined is why this difference exists. This effect could be used to trigger a response in the cell or just be a colonization factor. Future studies that can be done to study this question would be to test what factors affect adherence. It would be interesting to see if the presence of bile affects adherence. The bacteria could be grown in the presence of a non-lethal concentration of DCA prior to the addition to the tissue culture cells and see if there is a different response. It is also necessary to characterize the ability of the different

Helicobacter strains to adhere to various tissue culture cells. It would be helpful to measure the adherence of other *Helicobacters* that colonize the liver such as *H. hepaticus*. It would be expected that other bacteria that colonize the liver would also have increased adherence to the liver cell line.

Studying these phenotypic differences and using this bile assay, it will be possible to learn a lot about the pathogenicities of these species of *Helicobacters*. Studying the phenotypes will lead to studying the genotype of these organisms. It would be expected to find adherence factors, hepatotoxin, chemical transporter, or enzyme that allows the survival in the presence of bile. Creating isogenic mutants of these factors will allow the fulfillment of Koch's postulates on the molecular level. The most immediate use of this information is that it will be useful in the understanding of the bacterial etiology of hepatitis. It may also help in our understanding of idiopathic cases of hepatitis in other mammalian species as well as humans. Identifying these factors will also make it possible to look for these factors in other related species such as *H. pylori*. Even though they do not occupy the same organs, they may have similar mechanisms of pathogenesis involving inflammation. In a broader sense, the factors found in *H. bilis* may lead to new information on the evolution of the *Helicobacters*, or new diagnostic or epidemiological tools.

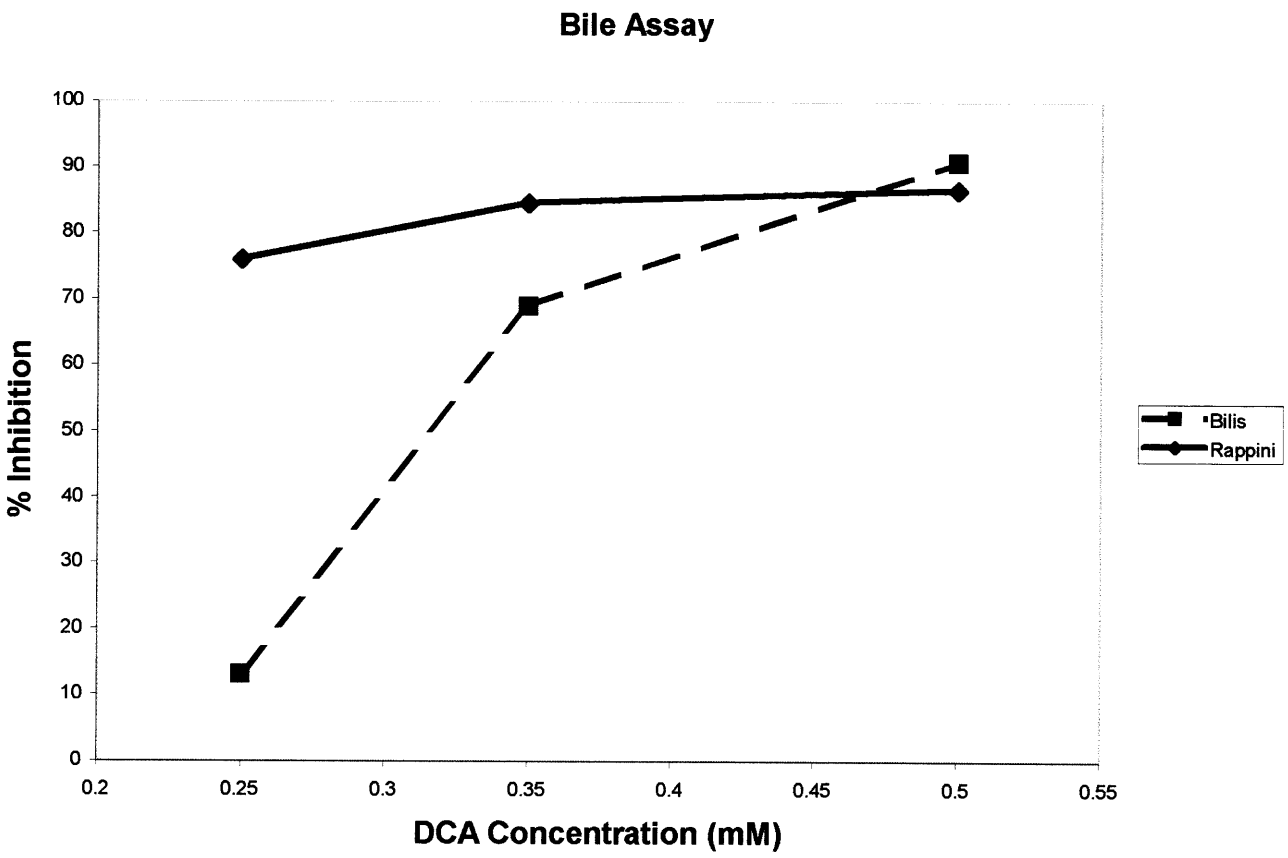

Figure 1: Preliminary bile assay of *H. bilis* and *F. rappini* 48hrs after inoculating with 1:100 dilution of starting culture. % Inhibition was calculated from OD_{600} levels. A significant difference in % inhibition can be seen at the 0.25 and 0.5 mM DCA concentration ($p<0.001$).

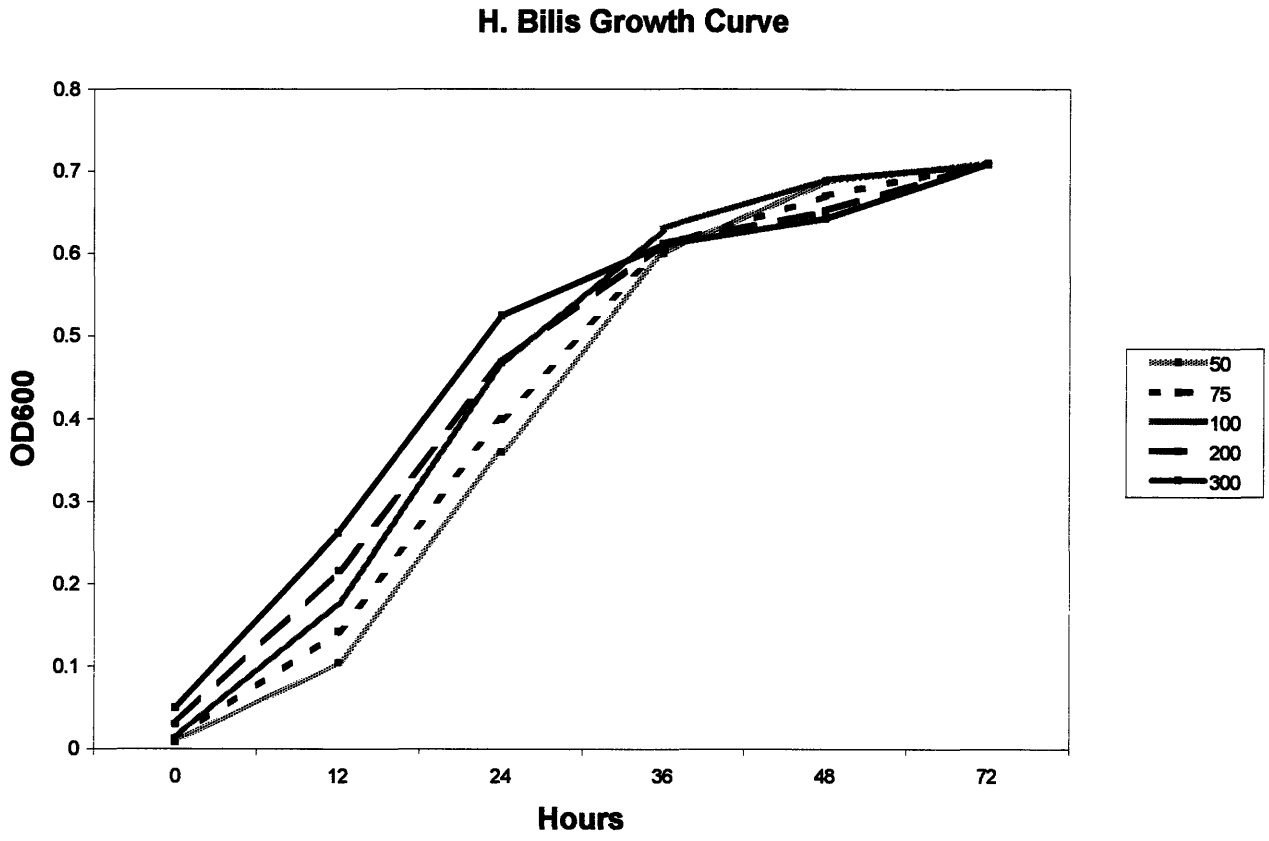

Figure 2: Growth curve of *H. bilis*. Samples were taken every twelve hours after carious levels of inoculation ranging from 1:50 to 1:300. A decrease in the length of the lag phase can be seen as the inocculum increases.

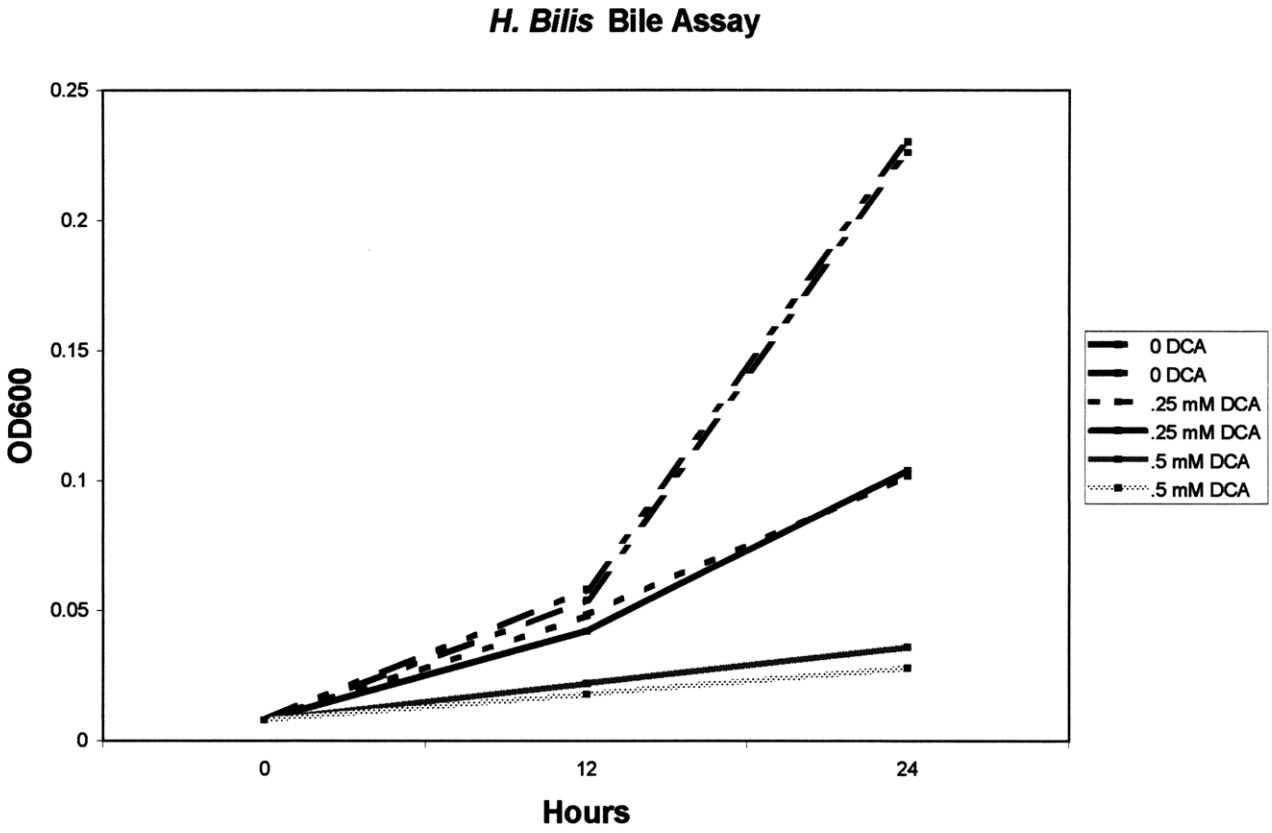

Figure 3: Bile assay using *H. bilis* and testing every twelve hours. % Inhibition was calculated and each level of DCA shows consistent responses to the bile acid.

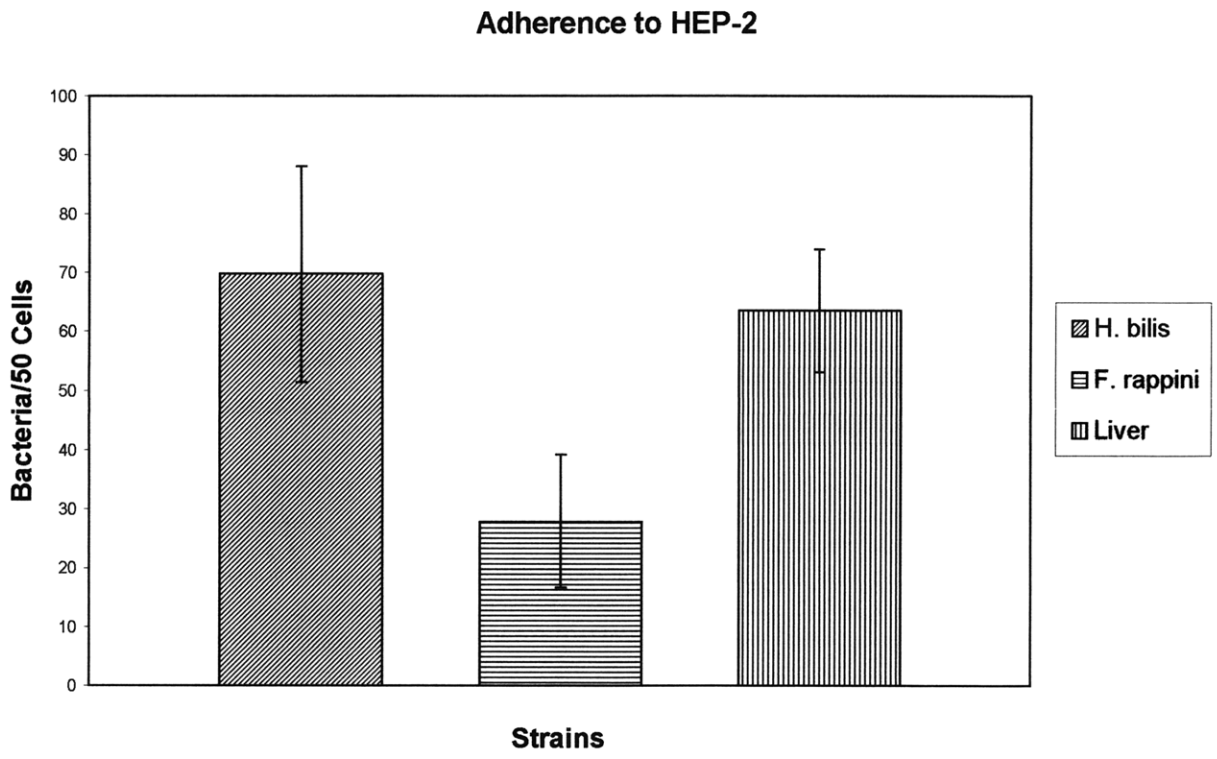

Figure 4: Adherence assay of *Helicobacters* and Hep-2 cells. Bacteria were counted in triplicate and each sample was done in triplicate. There is a significant difference between the level of adherence of *H. bilis* and *F. rappini* ($p<0.001$). There is no significant difference between a lab and a mouse passaged strain of *H. bilis*.

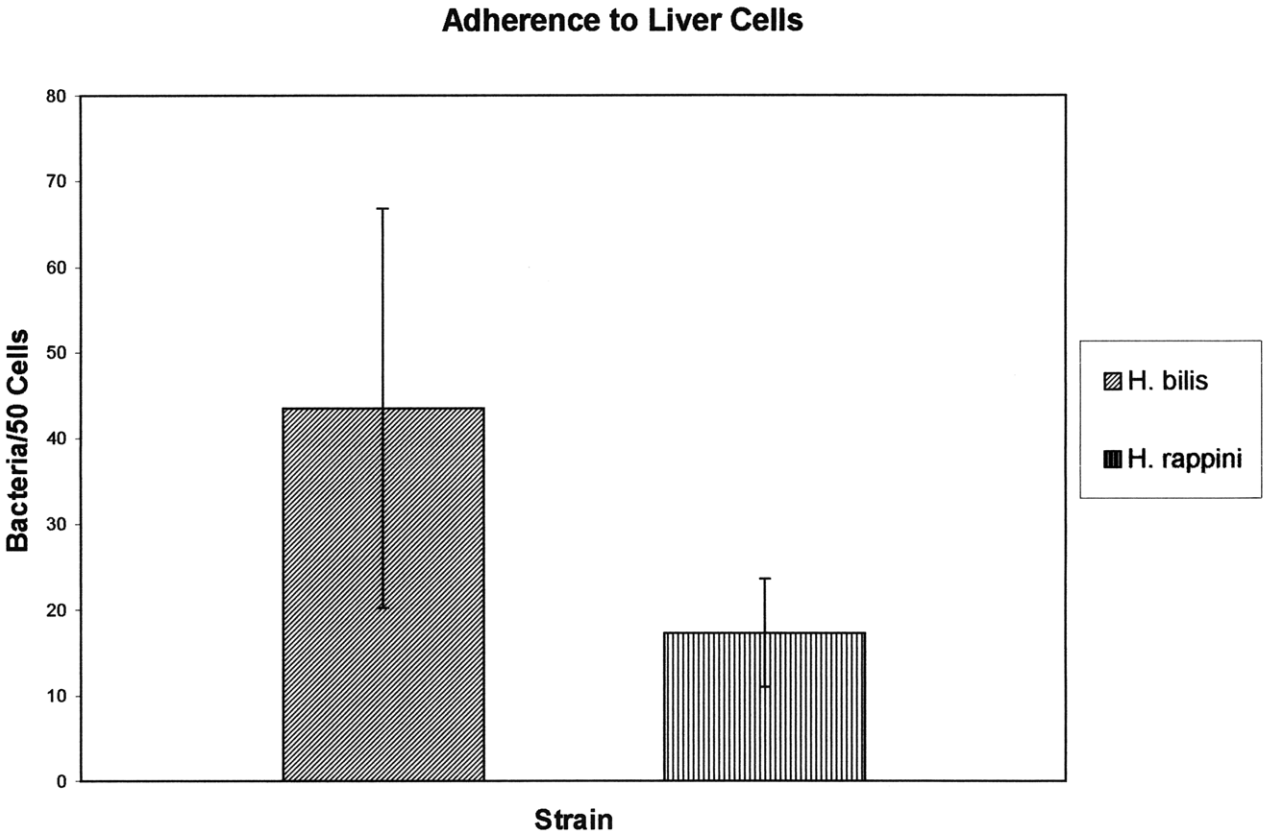

Figure 5: Adherence assay of *Helicobacters* and BNLC.2, a liver cell line. The bacteria were counted in triplicate and samples were tested in triplicate. There is a significant difference between these two *Helicobacters* ($p<0.001$).

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