Analysis of the antibiotic susceptibility and culture conditions of *Helicobacter cetorum*, a seaborne gastric pathogen

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Abstract

The *Helicobacter* genus is a diverse group of bacteria with many species that typically cause gastrointestinal disease in a variety of mammalian hosts. *Helicobacter pylori* is a well-studied pathogen in humans, is highly prevalent in the population, and causes gastritis, gastric ulcers, and carcinomas. *Helicobacter* spp. are naturally resistant to several antibiotics, and many *H. pylori* genes that are involved in antibiotic resistance have been identified and sequenced. Clarithromycin, metronidazole, and levofloxacin are common antibiotics used to treat *H. pylori* infections. Less is known about *Helicobacter* spp. that infect marine mammals, and *Helicobacter cetorum* is the only species that infects and causes gastric pathology in species within Cetacea, an order including dolphins, whales, and porpoises. *H. cetorum* is one of *H. pylori*'s closest relatives. To date, very few publications have been released with studies of *H. cetorum* in the lab; most studies have focused exclusively on diagnosing infection and determining tissue pathology. In this study, we aimed to expand the existing knowledge on *H. cetorum*. In addition to analyzing certain growth condition preferences, we performed a series of tests to determine its susceptibility to the three aforementioned antibiotics commonly used to treat *H. pylori*. The bacteria was found to be sensitive to clarithromycin and levofloxacin, but resistant to metronidazole. These results may have clinical significance, as *in vitro* antibiotic susceptibility is directly correlated with *in vivo* antibiotic susceptibility, and the results can be used by marine veterinarians to better treat these intelligent, beautiful animals.

Introduction

  The microbiome, all of the microorganisms in a particular environment, performs many different functions within its host organism. For example, bacteria residing on the skin aid in protecting the body from infection by other, more harmful microorganisms (Grice, 2011). In the gastrointestinal system, certain bacteria are essential for proper digestion and work to break down complex carbohydrates as well as other macromolecules. More recent research even suggests that the gut microbiome impacts other organs and systems such as the immune system and the brain (Barko, 2017). While resident microorganisms that reside in the gastrointestinal tract are usually beneficial, pathogenic microbes may be introduced, which cause harm to their host. Some are true pathogens, while others are opportunistic pathogens. True pathogens are microorganisms that always cause disease, and opportunistic pathogens are microorganisms that may cause disease if the host's body is put under some kind of stress, such as another illness or injury.

  The bacterial genus *Helicobacter* is generally comprised of true pathogens within mammalian hosts, and is thus of clinical importance. Many *Helicobacter* spp. were originally classified with the *Campylobacter* genus, but genomic sequencing revealed some species were different enough to name a new family. The name "*Helicobacter*" means spiral rod. Some general characteristics are shared by all *Helicobacter* species, such as a Gram negative cell wall structure and the presence of sheathed flagella, allowing for motility. In liquid media, these bacteria have external glycocalyx, which is a layer of of carbohydrates surrounding the cell membrane. While there is variability in the genus, many *Helicobacter* species cause gastrointestinal disease within their hosts (Owen, 1998). There are many *Helicobacter* spp. that

reside in the gastrointestinal tract of mammals; *Helicobacter cetorum,* the focus of this study, infects cetaceans and some pinnipeds (Suarez, 2010). *Helicobacter acinonychis* lives in big cats (Menard and Smet, 2019). *Helicobacter felis* is a common bacterium found in cats and dogs (Van Den Bulck et. al, 2005). *Helicobacter allurogastricus* also affects cats, *Helicobacter mustelae* is found in ferrets, and *Helicobacter typhlonius* colonizes mice (Cao et. al, 2016).

Helicobacter pylori is the best-studied *Helicobacter* species because it infects humans, colonizing the stomach lining and small intestine. To have a broad understanding of *Helicobacter* sp., we will now look at this well-studied bacterium in more detail. *H. pylori* causes stomach ulcers, and can even be a risk factor for adenocarcinomas (Parsonnet et. al, 1991). *H. pylori* can cause a spectrum of diseases and symptoms, and infection is very common in the human population. In 2015, approximately 4.4 billion individuals were infected with *H. pylori* worldwide; Africa had the highest prevalence, with 70.1% of the population affected (Hooi et al., 2017). It is a microaerophile, meaning it survives best in an environment with a small amount of oxygen and higher than normal levels of CO_2; it is a neutralophile, as it prefers a neutral pH between 5.5-8.5 when grown in the lab. Essential to its survival in the acidic environment of the stomach, is an enzyme called urease (Owen, 1998). The stomach has a very low pH (1-3.3) in order to digest food (Koziolek, 2015), and not many bacteria are able to withstand such a low pH (Owen, 1998).

Many different types of antibiotics are used to treat *H. pylori* infections. Sometimes, triple antibiotic therapy is used because *H. pylori* is naturally resistant to several classes of antibiotics. The three most common antibiotics used against an *H. pylori* infection are clarithromycin, metronidazole, and levofloxacin. *H. pylori* displays a 5-10% resistance rate to

clarithromycin and a 5-50% resistance rate to metronidazole (Maeda & Yoshida, 2001). There is also some resistance to levofloxacin (Wang et. al, 2018).

Clarithromycin is in the macrolide class of antimicrobials (Peters, 2012), and macrolides function by inhibiting essential protein synthesis by binding to the P site of the ribosome (Macrolides). The drug's effectiveness depends on its ability to bind to the cell's 50S bacterial ribosomal subunit. Mutations in the peptidyltransferase region of the DNA sequence prevent clarithromycin from binding to the subunit, thus causing resistance to the antibiotic (Francesco et al., 2011). The 23S rRNA gene mutations that cause clarithromycin resistance have been completely sequenced (Versalovic et. al, 1997).The minimum inhibitory concentration (MIC) of clarithromycin is relatively low, averaging around 0.016-0.5 mg/L. For clarithromycin, if a population has an MIC of greater than one mg/L, that population is considered resistant to the antibiotic (Francesco et al., 2011).

Metronidazole is a nitroimidazole antibiotic (Freeman, 2012), and it functions by unwinding and destroying the cell's DNA (Leiros, 2004). Amniotic radical nitro groups are reduced (given electrons), and these oxygenless radicals are toxic to DNA's structure. Metronidazole can also have an effect on the electron transport chain, jeopardizing the cell's ability to create ATP. The *rdxA* gene, which causes metronidazole resistance, has been heavily studied and fully sequenced (Jenks et. al, 1999). The MIC for this drug averages around 0.5-2 mg/L, with resistance being defined as an MIC above eight (Francesco et al., 2011).

Levofloxacin, a fluoroquinolone antibacterial (Davis, 2012), inhibits the repair of DNA by affecting the enzymes topoisomerase IV and DNA gyrase (Fookes, 2018). It does this by binding to subunit A of DNA gyrase. This can inhibit DNA and sometimes even RNA synthesis.

At high doses, levofloxacin is bacteriostatic, meaning it can halt cellular division. Mutations in the *gyrB* gene allow resistance to levofloxacin (Miyachi et. al, 2006)The MIC for levoflocacin averages around 0.25-0.50 mg/L, and populations are considered resistant if the MIC is above one (Francesco et al., 2011).

The focus of this study, *Helicobacter cetorum*, has only recently been discovered and unlike *H. pylori*, it has been studied very little. *H. cetorum* colonizes the stomachs of cetaceans (dolphins, whales, and porpoises) and some pinnipeds, such as the harp seal (Suarez, 2010), and *H. cetorum* has been isolated from and identified in more than thirty cetacean species (Harper, 2002). *H. cetorum* is hypothesized to be an opportunistic pathogen because it may reside in the gastrointestinal tract of healthy animals in small amounts, and if the animal's immune system is otherwise compromised, the bacterial population could increase and the animal could get sick (Goldman, 2011). *H. cetorum* often causes esophageal or forestomach ulcers, and can cause a myriad of related symptoms including loss of appetite, lethargy, regurgitation, and gastric fluid (Harper, 2002).

H. cetorum is most closely related to *H. pylori* and *H. acinonychis*. It is estimated that *H. pylori* and *H. cetorum* diverged from one another genetically about 600,000 years ago. Interestingly, cetaceans evolved around five million years ago, signifying that the bacterium did not evolve alongside its host species (Menard and Smet, 2019). *H. cetorum* is one of only two *Helicobacter* spp. to be isolated from marine mammals, with the other being *Helicobacter enhydrae,* which colonizes sea otters (Shen et al., 2017).

H. cetorum grows in a microaerobic environment, and prefers a temperature of 37-42 degrees Celsius. *H. cetorum* measures 0.6 by 4 micrometers. It is a fusiform- shaped bacterium

with a bipolar single flagellum. It is positive for urease, which is likely its primary acid-resistance mechanism. It grows on tryptic soy agar (TSA) infused with sheep blood serum, but no nutrition studies have been performed to date. *H. cetorum* is moderately susceptible to the antibiotic nalidixic acid, but published information about its antimicrobial susceptibility pattern is limited to that single antibiotic (Harper, 2002).

There is a large amount of genetic variation within *H. cetorum* strains. Strain MIT 99-5656, isolated from the stomach of a beached Atlantic white-sided dolphin (*Lagenorhynchus acutus*), and strain MIT 00-7128, isolated from the feces of a captive Beluga whale (*Delphinapterus leucas*), were compared to one another in one study by Kersulyte et al. They found that there was only sixty percent similarity between the two strains; comparatively, *H. pylori* strains typically share at least ninety percent of their DNA sequences. There were 411 proteins coded for in the whale strain that were not coded for in the dolphin strain, and 346 proteins coded for in the dolphin strain that were not coded for in the whale strain. One gene in the whale strain has been identified in other *Helicobacter* species, but is not found in *H. pylori* or the dolphin strain of *H. cetorum*. The genetic difference between these two strains led these researchers to question whether they should be grouped together as a species, or if there are two distinct species of *Helicobacter* colonizing cetacean stomachs (Kersulyte et al., 2013).

H. pylori and *H. cetorum* also share some potential virulence indicators. *VacA* is a gene found in *H. pylori* that contributes to the bacterium's pathogenicity; this gene is not found in any other *Helicobacter* species, but was found in *H. cetorum*. *H. pylori*'s gene for transformation was identified in both *H. cetorum* strains, indicating that *H. cetorum* populations can evolve rapidly

due to horizontal gene transfer. This could be dangerous in terms of developing antibiotic resistance within a population (Kersulyte et al., 2013).

H. cetorum infections may be quite common. The bacterium was identified in more than fifty percent of the dolphins tested in Sarasota Bay, FL, USA by the Sarasota Bay Dolphin Research Program (Harper, 2003). While this is not a representative sample of cetaceans worldwide, it is cause for concern. The symptoms are serious and can result in death from lack of proper nutrition if severe enough. This bacterium is important for many marine mammal populations and extremely understudied in terms of its laboratory growth and antimicrobial susceptibility, which is essential information for treatment of infected animals in captivity.

We chose to analyze some laboratory growth condition preferences of the bacterium to aid future research. In addition, because antibiotic susceptibility patterns may be of great interest to veterinary care providers seeking to treat infected cetaceans/pinnipeds in captivity, we tested *H. cetorum* against commonly prescribed classes of antibiotics in order to gain an understanding of which types of antibiotics are most effective. We tested three antibiotics commonly used to treat *H. pylori*--clarithromycin, metronidazole, and levofloxacin--and we used the standard Kirby-Bauer antibiotic susceptibility test method (Bauer, 1959).

Methods

  H. cetorum was cultured using both liquid and solid media. In both cases, the media was kept in a gas jar containing a Campy Pak following inoculation, and the jar was placed in a 37°C incubator (BD GasPak, Becton, Dickinson and Company, Franklin Lakes, NJ). Liquid media was based on culture conditions used for *H. pylori*, using 4.75 mL broth and 0.25 mL fetal bovine serum (FBS)--95% media, 5% FBS. Both tryptic soy broth and marine broth were tested in combination with FBS . Bacterial cells were swabbed using the hard tip of a sterile cotton swab, then the swab tip was brushed in the liquid to scrape off the cells.

  H. cetorum was grown initially on premade blood agar plates, before transitioning to individually prepared tryptic soy agar plates inoculated with sheep blood in order to maximize the freshness of the nutrients provided to the bacteria. 250 mL of media was made at one time. This was done by autoclaving a solution of 10.00 grams of TSA powder and 237.5 mLs of distilled water, then allowing the liquid to cool to approximately 55° C while stirring frequently. Once at the desired temperature, 12.5 mL room-temperature defibrinated sheep blood was aseptically added to the flask, then stirred slowly, resulting in media with 5% sheep blood by volume. As soon as the mixture appeared homogenous, the liquid was carefully poured into plates then allowed to solidify. The plates were stored at 4°C to minimize the risk of contamination.

  The Kirby-Bauer method of testing antibiotic susceptibility is standardized and is widely used. This method employs disc diffusion, opposed to the broth dilution or agar dilutions that are also sometimes used to test antibiotic susceptibility. The disc diffusion is beneficial because one can test multiple antibiotic compounds on a single agar plate (Biemer, 1973).

First, many plates were continuously streaked with bacteria. After 2-3 days of growth, as many cells as possible were gathered using the hard tip of a sterile cotton swab. Approximately two and a half continuously streaked plates were used to create one bacterial lawn. After gathering the cells, the swab tip was placed into a 1.0 mL Eppendorf tube with 1.0 mL 0.9% sterile saline and twisted to ensure the cells came off into the liquid. Once all the tubes had been prepared accordingly, they were centrifuged for 5 minutes at a speed of 12,500 RPM. Following centrifugation, almost all of the saline was carefully pipetted from the tube, leaving the saline with the bacteria at the bottom. 50 microliters of fresh saline was then added to each tube, then the tube was vortexed to ensure even redistribution of the bacterial cells throughout the liquid.

Each trial consisted of creating 4 lawns, and each tube yielded one lawn. Once all the tubes had been prepared, 60 microliters from each tube were pipetted onto plates. The liquid was then spread aseptically using a cell spreader. Once the liquid had been absorbed, the antibiotic discs were placed. One plate received a 15 mcg clarithromycin disc, one an 80 mcg metronidazole disc, one a 5 mcg levofloxacin disc, and the other did not receive a disc as a control (BD BBL Sensi Disc, Becton, Dickinson and Company, Franklin Lakes, NJ).

The plates were checked after 48 hours. The diameters of the zones of inhibition were measured in millimeters using a standard ruler. These numbers were then compared to the standards set by the Clinical and Laboratory Standards Institute to determine whether the bacteria was susceptible or resistant to that particular antibiotic (CLSI, 2019).

Results

This study yielded interesting results in regards to how the bacterium grows. Despite multiple culture attempts, *H. cetorum* did not grow in liquid media throughout this study. This

was confirmed via looking at a sample under the microscope. In addition, the premade plates that were used at the beginning of the study were several months old, and the bacteria was not growing efficiently on those plates. Once fresh plates were made, the bacteria appeared to grow better.

H. cetorum was sensitive to both clarithromycin and levofloxacin. The zone of inhibition was measured to be 60 mm in diameter for both trials with clarithromycin. The first trial with levofloxacin yielded a zone of inhibition diameter of 64 mm, while the second trial yielded a zone of inhibition diameter of 58 mm. Interestingly, the plates testing metronidazole susceptibility did not present with any zones of inhibition at all for either trial, indicating that *H. cetorum* is resistant to this particular antibiotic.

Discussion

The difficulty of growing *H. cetorum* in liquid media does not have a defined cause. Liquid media is stored in a vial, and oxygen is available in varying amounts throughout the vial. It is possible the oxygen was not available in exactly the amount it needed to be for the bacterium to grow properly in the vial. It is also possible the nutrients were somehow not distributed throughout the liquid properly, affecting the bacteria's ability to survive.

Additionally, its willingness to grow effectively on only fresh blood agar plates is very interesting. Premade plates ordered online typically come with an expiration date several months later, and they are stored in the fridge to preserve them and to prevent contamination. One can also often continue to use plates for a while after the expiration date. However, *H. cetorum*'s growth rate appeared to decrease as the plates got older and older. Finally, the bacteria ceased to

appear on the plates at all. The materials to make fresh blood agar plates were ordered at that point.

Once the bacteria was being cultured on fresh plates, it grew at a moreconsistent rate. The bacteria were clearly visible on the plates. It could just be that the bacteria thrived having fresh nutrients, or it could have to do with the freshness of the blood in the plate. As *H. cetorum* typically lives in the G.I. tract of an animal, it needs the nutrients from blood to survive; this is also standard for fastidious microbes in general. This better understanding of the growth habits of *H. cetorum* could aid future research by allowing for more efficient laboratory growth, and therefore more efficient and reliable research.

In terms of the susceptibility to antibiotics, it was not a surprise that *H. cetorum* was susceptible to clarithromycin and levofloxacin. It was surprising, however, that the zones of inhibition were so large; they were significantly larger than the zones of inhibition of most bacteria/antibiotic combinations. This would indicate that *H. cetorum* is extremely sensitive to both clarithromycin and levofloxacin. According to our results, *H. cetorum* is resistant to metronidazole. This is a bit shocking, considering how susceptible it was to the other two antibiotics. This indicates that the metronidazole was somehow blocked from getting to the cell's DNA, or the bacterial cell did not allow the nitro group to be reduced, or some other mechanism that would allow for resistance against this antibiotic. As mentioned earlier, metronidazole is the antibiotic that has the highest prevalence of resistance in *H. pylori*, so it is unsurprising that, if *H. cetorum* were to display resistance to an antibiotic, it would be metronidazole (Maeda & Yoshida, 2001).

The resistance to metronidazole could provide information about *H. cetorum*'s DNA. As mentioned earlier, *rdxA* has been identified in *H. pylori* as the gene causing resistance to metronidazole (Jenks et al., 1999). It is possible that *H. cetorum* has the same or a similar gene allowing for resistance to this antibiotic.

Project Significance

Very little research has been done on this bacterium, and the outcomes from our study should provide a solid foundation for future research as well as modify existing treatment protocols for captive cetaceans with *H. cetorum* infections. In more thoroughly studied bacterial pathogens, antibiotic susceptibility patterns have been examined in detail. This study could be the first step towards providing some of that foundational information about *H. cetorum,* and it has the potential to greatly benefit the cetaceans and pinnipeds affected by the pathogen through improved veterinary care.

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