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Characterization of HmuY-Like Heme-Binding Protein of *Leptospira interrogans*

Antonia Merta
Research Mentor: Dr. Jesus Segovia

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Abstract

Leptospira interrogans is a pathogenic spirochete that causes the disease known in both the medical and veterinary worlds as Leptospirosis[1]. Canines are commonly infected by leptospires, and in fact, there are vaccines against Leptospirosis for dogs[2]. The canine vaccines against Leptospirosis contain serovars canicola, icterohaemorrhagiae[3], grippotyphosa, and pomona[2]. Though there is a vaccine against Leptospirosis for humans, as well, it is not widely available[2]. The first vaccines against Leptospirosis contained killed leptospires in a medium partly composed of serum, which often caused undesirable side effects[2]. The vaccine in current use is cultured in a medium that does not contain proteins and therefore, does not cause such severe side effects[2]. However, because the immune response to infection by leptospires is antibody-mediated[4], it is necessary that the same or an extremely similar serovar to the one that is infecting humans be used in the vaccine, which is difficult to ensure[2].

Leptospirosis is a zoonotic disease in that it is spread to humans by animal carriers[5]. Furthermore, it is currently the most pervasive zoonosis in the world[6]. On the occasion that contact between the two parties is indirect, humans are infected via soil or water that has been contaminated with the urine of infected animal hosts[5]. Dogs are usually infected via the indirect route[7]. Leptospires enter the body through the mucous membranes of the nose, mouth, and eyes, or through wounds in the skin[5]. Once they have entered successfully, the leptospires spread throughout the body via the blood vessels[5]. As with most diseases, and especially because Leptospirosis is a disease of the blood, it comes in many forms[2]. Even the symptoms of the mild form of infection are most undesirable. These may include but are not limited to severe headaches that may be related to inflammation of the meninges, stomachaches, muscle aches, chills, redness of the eyes, and skin rash[2]. More serious forms of Leptospirosis can be debilitating, yet it is not financially feasible for all infected humans or dogs to receive necessary

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treatments. If left untreated, Leptospirosis can lead to organ failure, which may result in a prolonged and painful death[5]. In its more severe forms, Leptospirosis can cause myocarditis, which may lead to heart failure, kidney failure, liver failure, and pulmonary hemorrhage, among other serious possible complications[2]. Leptospirosis has even been known to cause the spontaneous abortion of fetuses in affected pregnant women[2]. Though the disease caused by *L. interrogans* is well understood, the spirochete's exact mechanisms of disease relating to virulence factors and implicated proteins are still under continuous research[2].

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Dedication

To my oldest brother, David Merta, who showed the most interest in my Leptospirosis research and encouraged me to keep working, and whose joyful love of learning about anything and everything is an inspiration to all students of life.

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Acknowledgments

I thank St. Mary's University for providing me with the opportunity and the means for completing this thesis and the scientific research that went into it. I thank the St. Mary's University science department, including all of my biology and chemistry professors who opened my mind to the beautiful fascinations of science and taught me to enjoy making novel discoveries through research and experimentation. I thank the St. Mary's University honors program and associated professors, without whom I would not be adequately prepared nor possess the opportunity to write this thesis. I thank my father, Tony Merta, and my fiancé, Cole Holub, who have fully supported me and have been selflessly patient with me through my most trying days. I thank my local veterinarian, mentor, and friend, Dr. Joe Jaksik, who has patiently taught me about veterinary medicine over the last six years, inspired me to work towards becoming a veterinarian, and gave me the idea to research Leptospirosis. Lastly, and especially, I thank Dr. Jesus Segovia for the guidance, help, encouragement, generosity, and kindness he has shown me throughout his mentorship; I could not have completed this thesis without him.

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I. About *Leptospira interrogans*

Classification

Leptospirosis, the disease branded by jaundiced skin and eyes, as can be seen in Figure 1, in addition to fever, sub-clinically, and by organ failure in its most deadly stages, was first characterized by Adolf Weil in 1886[8]. However, leptospires themselves were first described by Arthur Stimson in 1907, who silver stained clumps of the spirochetes in the kidney tubules of an individual who was thought to have died from yellow fever[2]. Stimson gave them the name *Spirochaeta interrogans* based on the question mark shape they displayed when viewed beneath a microscope's lense[9]. In 1915, Inada and Ido from Japan identified *Leptospira ictero-hæmorrhagiæ* as the causative agent of Weil's disease, which was confirmed by German physicians, Uhlenhuth and Fromme, and Hübener and Reiter[8], who studied the disease that infected German soldiers entrenched in French soil during World War I[2]. Leptospires were not initially identified as the cause of the signs and symptoms now known to characterize the disease we call Leptospirosis. However, there are historical records of the leptospiral disease that plagued rice farmers in ancient China, as well as was long described by the Japanese as *akiyami*, (autumn fever)[2].

There are currently 35 species and 90 strains that fall under the genus *Leptospira*[9]. In 1907, Stimson initially divided the genus into a pathogenic group, *Leptospira interrogans sensu lato,* and a non-pathogenic group, *Leptospira biflexa sensu lato*[9]. However, the genus is now divided into three lineages based on pathogenicity[9]. Based on this new classification, Spirochetes are now classified as being saprophytic, intermediate, or pathogenic[9].

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[Photograph]. (2020, December 9). *Jaundice "Yellow Skin" In Dogs Causes and Treatments*. Retrieved from https://vetwork.co/galaxy/en/jaundiceyellow-skin-in-dogs-causes-and-treatments/

Figure 1: A canine infected with Leptospirosis experiences yellowing of the eyes, due to the corresponding jaundice.

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Structure

Leptospira interrogans is a spirochete that has the appearance of a question-mark shaped worm when seen through the lens of a microscope,[6] as shown in Figure 2. Although it has the appearance of a worm, it is important to keep in mind that *L. interrogans* is not a parasite, but rather, a type of endoflagellar bacteria. Spirochetes of the *L. interrogans* species possess two flagella within the axial filament of the periplasmic space between the cytoplasmic and outer membranes[10]. Each flagellum extends from one subterminal end without becoming intertwined with the other[10], and together their 360° clockwise or counterclockwise motion leads to the corkscrew movement characterized by the spirochete[11]. Other pathologically common spirochetes include the agents of syphilis and Lyme disease, *Treponema pallidum pallidum* and *Borrelia burgdorferi,* respectively[11]. Some scientists reportedly are considering the possibility that the corkscrew motion itself contributes to the ability of these spirochete pathogens to infect tissues[11]. *Helicobacter pylori*, the non-saprophytic bacterium often responsible for gastric ulcers, has been known to utilize a corkscrew motion during its penetration and subsequent colonization of the gastric mucosa[12].

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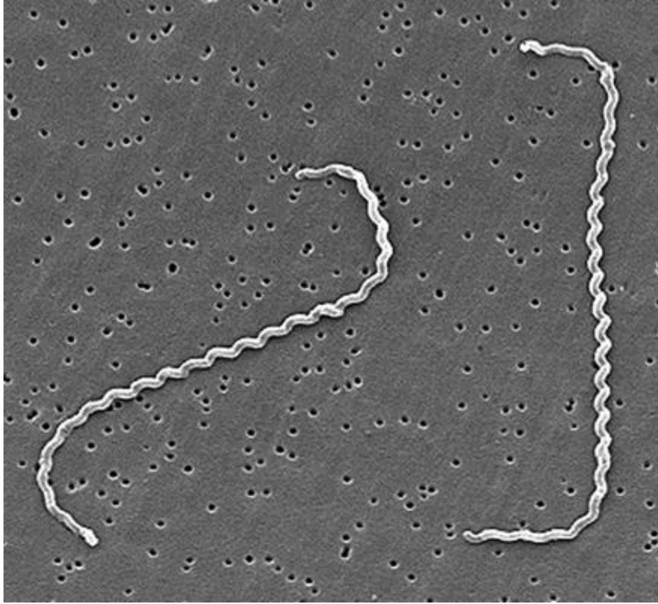

[Scanning electron micrograph of *L. interrogans* serovar icterohaemorrhagiae strain RGA bound to a 0.2-µm membrane filter. Reproduced from reference 625a with permission from the publisher.]. (1969, December 31). Retrieved from https://cmr.asm.org/content/14/2/296/F1

Figure 2: This scanning electron micrograph shows the distinct question-mark shape associated with *Leptospira interrogans*.

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L. interrogans is unique in that it shares features of both Gram-negative and Gram-positive bacteria, though it is classified as Gram-negative[6]. Similar to Gram-positive bacteria, the cytoplasmic membrane of *L. interrogans* is closely associated with its peptidoglycan cell wall, though it still possesses a periplasmic space between the two[13]. Like other Gram-negative bacteria, its flagella possess four protein rings[11]. Because it contains an outer membrane in addition to its inner cytoplasmic membrane, the layer of peptidoglycan that it possesses is rather thin compared to purely Gram-positive bacteria[11]. This thin layer of peptidoglycan is composed of alternating molecules of the sugars, N-acetylglucosamine, (NAG), and N-acetylmuramic acid, (NAM), attached to tetrapeptide cross-bridges[11]. It is located in the periplasmic space, in addition to the flagella within the axial filaments there (as previously noted), between the inner and outer membranes[11]. This periplasmic space contains water, nutrients, and enzymes that are involved in molecular transport[11]. The outer membrane of the spirochete's cell wall is composed of an inner leaflet of phospholipids and proteins, and an outer leaflet of lipopolysaccharide (LPS)[11]. LPS is composed of a sugar and Lipid A, which is toxic to host cells upon the destruction of the spirochete's outer membrane[11]. This toxicity upon release results in negative reactions including, but not limited to fever, shock, and inflammation[11]. This Gram-negative feature of the spirochete results in a reduced effectiveness of antimicrobial drugs since they must be given in small dosages[11].

Virulence Factors

As of 2020, there are twelve virulence factors known to assist *L. interrogans* in infecting its hosts[14]. Adenylate/guanylate cyclase, (AGC), increases the levels of cAMP in macrophages within the host cell, which causes a decrease in the amount of tumor necrosis factor alpha, (TNF-

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α), released[14]. This reduces the effectiveness of the inflammatory response occurring against *L. interrogans*[14]. ClpB/Hsp100 is a chaperone protein that may contribute especially to the survival of *L. interrogans* during a host immune response by way of its protein disaggregation ability, as well as its possible role in allowing leptospires to escape host phagosomes and continue infecting host cells[1415]. High-temperature protein G, (HtpG/Hsp90), is another chaperone protein whose role in infection of host cells by *L. interrogans*, though not fully understood, also may be related to assisting in adaptability and survival of the leptospire during an immune response mounted by its host[16]. FlaA2, FliY, FliM, and FcpA are unique structural elements of the flagella of *L. interrogans*[10171819]. As such, they contribute to the leptospire's ability to penetrate and spread throughout the host[10171819]. KatE is a periplasmic catalase that may assist *L. interrogans* in escaping from host cell macrophages and neutrophils via detoxification of host cell reactive oxygen species, (ROS), such as H_2O_2[20]. LB139 is a sensor protein that regulates gene expression in *L. interrogans*, including regulation of those genes coding for proteins vital to its chemotaxis and motility abilities[21]. LoA22 is an OmpA-like outer membrane protein of *L. interrogans* that may contribute to its acute infection methods[22]. LPS is a component of the cell wall of *L. interrogans* that can trigger inflammation in the host[14]. In addition, LPS has been found to contribute to the leptospire's capacity to survive temperature changes undergone during the transition between survival outside of a host and host cell infection[14]. LruA is an inner membrane lipoprotein of *L. interrogans,* which interacts with ApoA-I involved in a host cell's immune response[23]. LruA reduces ApoA-I binding to *L. interrogans*, which otherwise induces bactericide[23]. Mammalian cell entry protein, (Mce), and collagenase A, (ColA), are outer membrane proteins of *L. interrogans* that are thought to be involved in the initial attachment and entry, as well as the spreading of the leptospires throughout a host[24]. Phospholipase C is a gene

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product of LB361, which, in addition to hydrolyzing phosphatidylinositol-4,5-bis phosphate, also plays a role in increasing the amount of free calcium ions present, which kills activated macrophages[25]. This, in turn, reduces the possibility of an effective immune response against *L. interrogans*[25]. Heme oxygenase, (HemO), is an enzyme that degrades heme, which assists *L. interrogans* in acquiring iron from hemoglobin within host blood cells[26].

Epidemiology

Rodents, especially mice and rats, as shown in Figure 3, serve as the primary host for pathogenic leptospires, including *L. interrogans*[8]. Carrier rodents shed the leptospires in their urine, contaminating water sources that are used by other animals and humans for drink or recreational activities[27]. Unlike most pathogenic strains of leptospire, *L. interrogans* can survive for long periods of time outside a host[6]. They, along with nonpathogenic leptospires, thrive in warm, moist conditions[27]. This makes the tropics and tepid bodies of water ideal habitats for *L. interrogans* to lie in wait for a host to infect[27]. Outbreaks of Leptospirosis among humans are correlated with flooding, especially in developing countries where sanitation quality is generally poorer[6]. Increased incidence of infection is also associated with water sports[6]. Farming is considered a risk factor for becoming infected with Leptospirosis, due to close interactions with the land and animals[6]. Furthermore, veterinary epidemiological studies have shown that the incidence of Leptospirosis among canines displays a clear, positive correlation with an increase in warm, rainy weather[27]. It is important to note that though dogs are often in contact with humans, they have not been conclusively proven to be vectors of transmission of Leptospirosis to humans[27].

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[There are three cases of a bacterial infection caused by rat urine, one of them fatal, in the Bronx according to New York City officials.]. (2017b, February 15).
Retrieved from https://6abc.com/leptospirosis-new-york-city-bronx-rat-urine/1756158/

Figure 3: Rats, such as those shown here, that are carriers of *L. interrogans* spread the bacterium by urinating in water sources used by other animals for drinking and by humans for recreational water activities.

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II. *L. interrogans* Infection in Humans

Infection Method

After exposure to *L. interrogans,* there is a seven to twelve day incubation period within the host[28]. The infection that follows is normally biphasic[28]. The first phase that succeeds the incubation period lasts for four to seven days and is characterized by mild symptoms[28]. This period is followed by a two-day break from fever[28]. Afterwards, the second phase ensues and may continue for up to thirty days, during which more severe symptoms begin[28]. During this stage, leptospires are commonly detected in the urine of infected hosts[5]. The pathogenesis of *L. interrogans* is still being researched, and therefore, not much is known about the spirochete's particular mechanisms of infection other than a few general details[6].

At the cellular level, leptospires first adhere to host cells by employing surface proteins, which recognize adhesive portions of the extracellular matrix or of the cell membrane itself[29,30]. Leptospiral LPS plays a significant role in the adhesion process through its adherence to the collagen, fibronectin, and laminin that comprise the host cell's extracellular matrix[31]. It has also been shown to play a role in host cell macrophage activation[32]. In addition, leptospiral immunoglobulin-like proteins play a role in the adhesion, colonization, and dissemination processes through their interaction with extracellular matrix proteins[33,34]. Furthermore, leptospiral SphH causes pores to form in host cells and SphA uses sphingomyelinase to promote cell membrane permeability and aid in the diffusion of *L. interrogans* cells[35,36]. After adhesion, leptospires have been known to bind to fibroblasts, macrophages, and endothelial cells throughout the host's body, in addition to kidney epithelial cells[37]. It has been shown that *L. interrogans* can avoid being eliminated by the host enacted complement cascade[38]. The leptospires accomplish this by recruiting host complement regulators, acquiring host proteases in

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order to cleave complement proteins on leptospire cell surfaces, and secreting their own proteases that inactivate complement proteins in the surrounding cellular environment[38]. Furthermore, leptospires release hemolysins Sph1, Sph2, Sph3, HlpA and TlyA to activate a host inflammatory response, which is largely mediated by the release of cytokines[39].

Host Immune Response

The host immune response against *L. interrogans* has been shown to be similar in dogs and humans[8]. The primary humoral response in both is led by antibodies of class IgM and IgG, the former of which presents first and also longest of the two[40]. Detection of IgM is most important in diagnosing Leptospirosis in canines[40]. Though IgM and IgG activity is important in the immune response of humans with Leptospirosis, recent experiments have demonstrated several other key factors that are likely also applicable to canine infection[41]. Leptospires can stimulate the Toll-like receptor 2 (TLR2) pathway, along with CD14 to stimulate the release of proinflammatory cytokines by macrophages via LPS engagement[32]. Cytokines and chemokines, and particularly serum mediators, arginine vasopressin, soluble serum stimulation-2, long pentraxin, and nitric oxide, are involved in the host immune response to *L. interrogans* infection[41].

(TNF-α) is a pro-inflammatory cytokine produced by macrophages, monocytes, and renal cells[42]. It has been implicated in blood vessel inflammation, hemorrhaging, liver, kidney, and even lung impairment, and death caused by Leptospirosis[42]. Interleukin 10 (IL-10) is an anti-inflammatory cytokine[42]. Its role in the host immune response against Leptospirosis is under continued investigation, but there is evidence that its over-stimulated production may lead to inappropriate inhibition of the Th1 inflammatory response[42]. IL-6 has both pro-inflammatory and

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anti-inflammatory characteristics[43], and has been implicated in the causation of severe pulmonary hemorrhage syndrome, which often leads to death as a result of *L. interrogans* infection[44]. IL-8, IL-6, granulocyte-macrophage CSF (GM-CSF), vascular endothelial growth factor (VEGF), monocyte chemoattractant protein-1 (MCP-1), and interferon-inducible protein 10 (IP-10) have further been associated with hemorrhaging and lung impairment during Leptospirosis[43].

Serum mediators are set apart from the cytokine and chemokine players in the host immune response to Leptospirosis because they are unique to *L. interrogans* infection[41]. Hyper-release of arginine vasopressin during *L. interrogans* infection causes renal impairment, which manifests itself through low blood sodium levels[45]. Furthermore, increased secretion of soluble ST2 (sST2), in the presence of IL-6, IL-10 and IL-8 have been associated with cases of Leptospirosis where fatal bleeding occurs[46]. Long pentraxin is involved in acute immune responses and is released by endothelial cells, epithelial cells, dendritic cells, and mononuclear phagocytes, among other human body cells[47]. It has been implicated, along with C-reactive protein, IL-8, and IL-6, in increased mortality associated with Leptospirosis[47]. Nitric oxide is associated with endothelial dysfunction during Leptospirosis, which can lead to vascular damage and in turn, organ damage[4849].

Similarity to *Borrelia burgdorferi*

It should be emphasized that as the structure of spirochetes is similar across different species, and by default, so too are the functions of certain proteins involved in their pathogeneses[11]. As was previously mentioned, the agents of Lyme disease and syphilis are both spirochetes like *L. interrogans*[11]. Though the agent of syphilis, *Treponema pallidum pallidum,*

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differs substantially in its infection methods[50], the agent of Lyme disease, *Borrelia burgdorferi,* shown in Figure 4, shares key pathogenic similarities with *L. interrogans*[51]. *B. burgdorferi,* like *L. interrogans,* employs the use of adhesins that bind to fibronectin, among other host cell extracellular matrix components[51]. *B. burgdorferi* can also evade the host immune response by resisting the complement cascade shown in Figure 5[51]. In addition, *B. burgdorferi,* too, stimulates an inflammatory host immune response via cytokines and chemokines[51].

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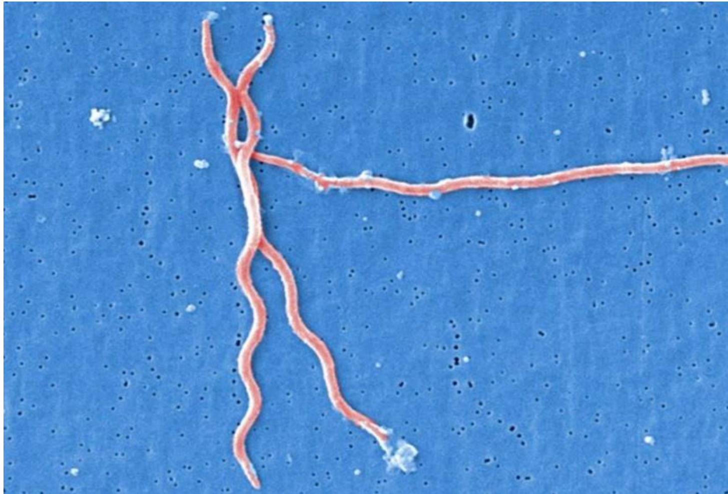

Carr, J. H. (n.d.). *Borrelia burgdorferi bacteria* [Photograph]. Retrieved from http://www.bacteriainphotos.com/Borrelia_burgdorferi.html

Figure 4: *Borrelia burgdorferi,* the agent of Lyme disease shown above, shares several structural and functional similarities with *L. interrogans.*

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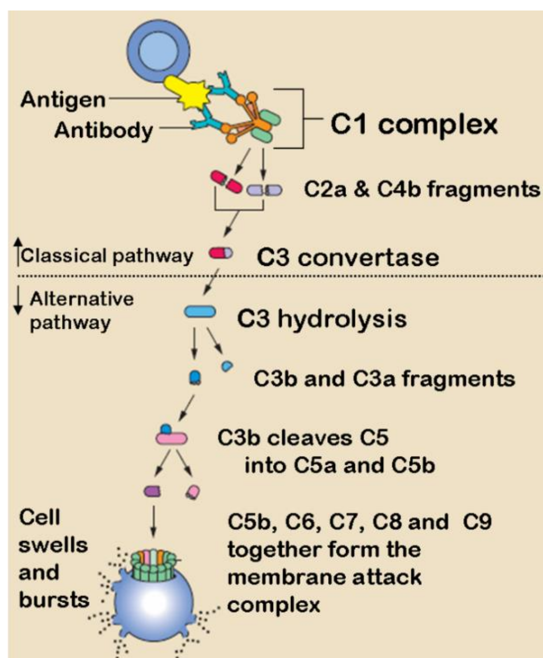

[Illustration]. (2020, August 13). *The Classical and Alternative Complement Pathways*. Retrieved from https://med.libretexts.org/Bookshelves/Anatomy_and_Physiology/Book%3A_Anatomy_and_Physiology_(Boundless)/20%3A_Immune_System/20.6%3A_Humoral_Immune_Response/20.6C%3A_Role_of_the_Complement_System_in_Immunity

Figure 5: Both *B. burgdorferi* and *L. interrogans* evade host immune responses by interrupting the natural progression of the complement cascade.

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III. Identification of a Novel Potential Virulence Factor of *L. interrogans*

HmuY Heme-Binding Protein's Essential Role in *Porphyromonas gingivalis*

Porphyromonas gingivalis, like *L. interrogans*, is a Gram-negative bacteria that is responsible for an assortment of diseases in humans, dogs, and cats, the most common of which is periodontitis[52]. Periodontitis, as shown in Figure 6, is characterized by painful inflammation of the gums, which may lead to tooth decay and oral tissue damage if left untreated[52]. As a pathogenic bacterium, *P. gingivalis* obtains nutrients from its host. One of the nutrients required for its survival is iron in the form of heme. Iron is also essential for many cellular processes such as photosynthesis, successful function of the electron transport chain, oxygen storage, and biochemically necessary reduction-oxidation reactions[52]. The HmuY protein plays a vital role in the successful acquisition of heme by *P. gingivalis*[52]. Once *P. gingivalis* proteases and hemolysins obtain heme from host cell hemoglobin or myoglobin (among other possible heme sources), bacterial heme carriers convey the free heme to HmuY protein, which passes it along to the TonB-dependent receptor, HmuR, which then transports the heme across the bacterial cell membrane[52]. In addition to the crucial role HmuY plays in iron acquisition, a study by Teresa Olczak, et. al, has provided evidence for the hypothesis that as a surface protein, HmuY may also play a vital role in biofilm formation during *P. gingivalis* infection[53].

As with all proteins, the structure of HmuY is directly related to its function in assisting with the acquisition of heme. HmuY is predominantly made up of β-sheets[52]. Its structure, as can be seen in Figure 7, is reminiscent of a right human hand whose thumb and fingertips entrap heme at four separate binding sites[52]. Once the heme is bound, HmuY forms tetramers to surround each occupied heme-binding site and prevent the host cells from accessing the heme[52].

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HmuY has been found to be resilient in nature; it is resistant to denaturation via trypsin, *P. gingivalis* proteases, or chemical or thermal means[52].

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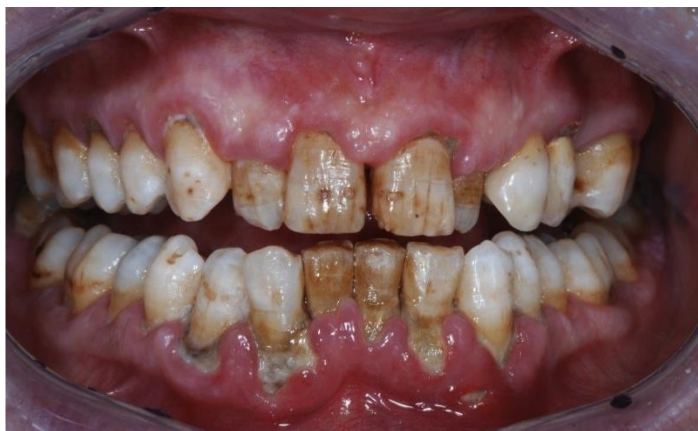
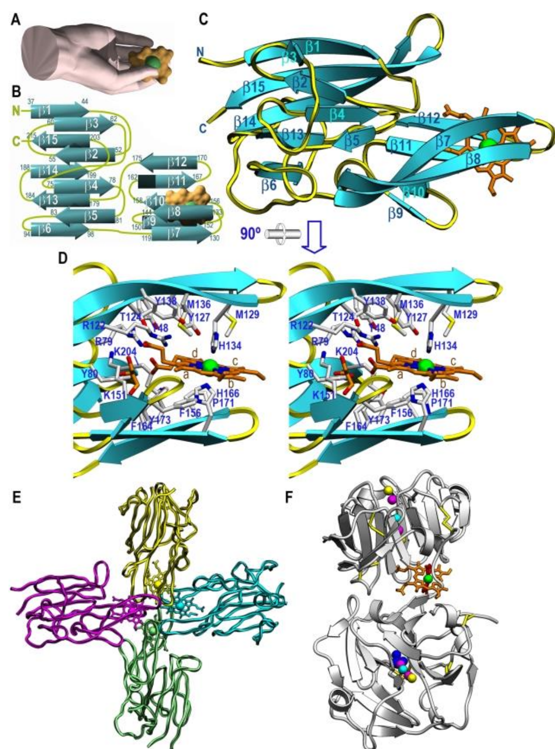


Studio Cozzolino. (n.d.). *How is periodontitis diagnosed?* [Photograph]. Retrieved from https://www.cozzolinodentaloffice.com/periodontitis/
Figure 6: Periodontitis is caused by the bacterial agent, *P. gingivalis.*



Wójtowicz et al. (2009, May 8). *Structure of holo-HmuY* [Illustration]. Retrieved from https://pubmed.ncbi.nlm.nih.gov/19424422/
Figure 7: This figure shows the unique structure of the HmuY heme-binding protein present in *P. gingivalis.*

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Presence of an HmuY-Like Heme-Binding Protein in *Leptospira interrogans*

As it turns out, within the genome of *Leptospira interrogans,* there appears to be the presence of a gene that encodes an HmuY-like heme-binding protein. We discovered this as a result of a lengthy research process that began with a look into canine Leptospirosis. Considering the danger that may be involved in working with a pathogenic organism known to also cause human disease, our attention was temporarily turned to the nonpathogenic, saprophytic spirochete closely related to the evolutionary predecessor of both nonpathogenic and pathogenic spirochete species. This saprophyte is known by the name of *Leptospira biflexa.* By reviewing the scientific literature on PubMed, we soon learned that *L. biflexa* possesses a third, unique replicon within its genome known as p74, as shown in Figure 8, which contains genes for 15 hypothetical proteins, some of which are similar to proteins in pathogenic leptospires[54]. This similarity was determined by running a protein Basic Local Alignment Search Tool, (BLASTp), on NCBI, as seen in Figure 9, for each of the fifteen proteins categorized as hypothetical at the time, (there are now only fourteen), in order to identify any possible matches in all other organisms known to man and documented there. This is when we discovered that a 254 amino acid protein previously classified as hypothetical on *L. biflexa*'s p74 plasmid has a roughly 30% identity match to an HmuY-like heme-binding protein within the genome of *L. interrogans.* It should be noted that since we first ran this BLAST, the *L. biflexa* hypothetical protein in question has since been renamed to "HmuY family protein," due to its undeniable similarity to HmuY-like heme-binding proteins in countless other *Leptospira* species.

Thus, our attention was turned back to *L. interrogans*. Instead of trying to determine the role of one of the hypothetical p74 proteins in the environmental survival of saprophytic *L. biflexa,* we now were determined to characterize this HmuY-like heme-binding protein's

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structure and therefore, also detail its role in successful infection of the host by *L. interrogans.* This protein may play a significant enough role in our leptospire's pathogenicity that it could be classified as an additional virulence factor. Similar to the HmuY heme-binding protein present in *P. gingivalis,* in *L. interrogans,* this protein may very well be vital to the bacterium's successful infection of a host cell[52]. Upon the completion of certain experimental methods that will be listed in the following section, it is our hope that this may one day be determined.

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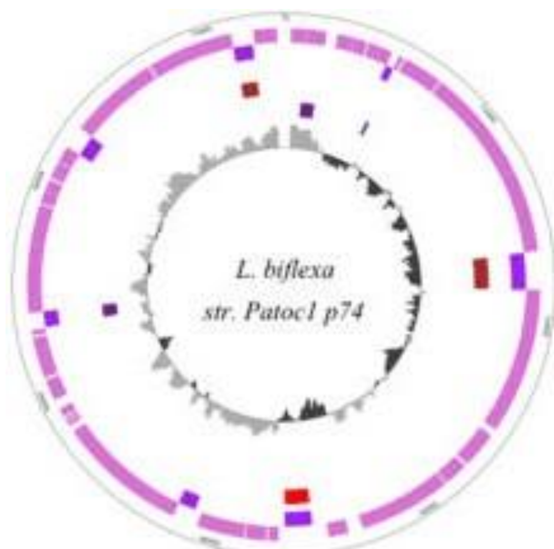

[Illustration]. (2008, February 13). *Circular maps of the three L. biflexa replicons.* Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2229662/

Figure 8: This figure displays the circular map of the p74 plasmid that resides in *Leptospira biflexa* and initially led us to the HmuY-like heme-binding protein present in the genome of *L. interrogans.*

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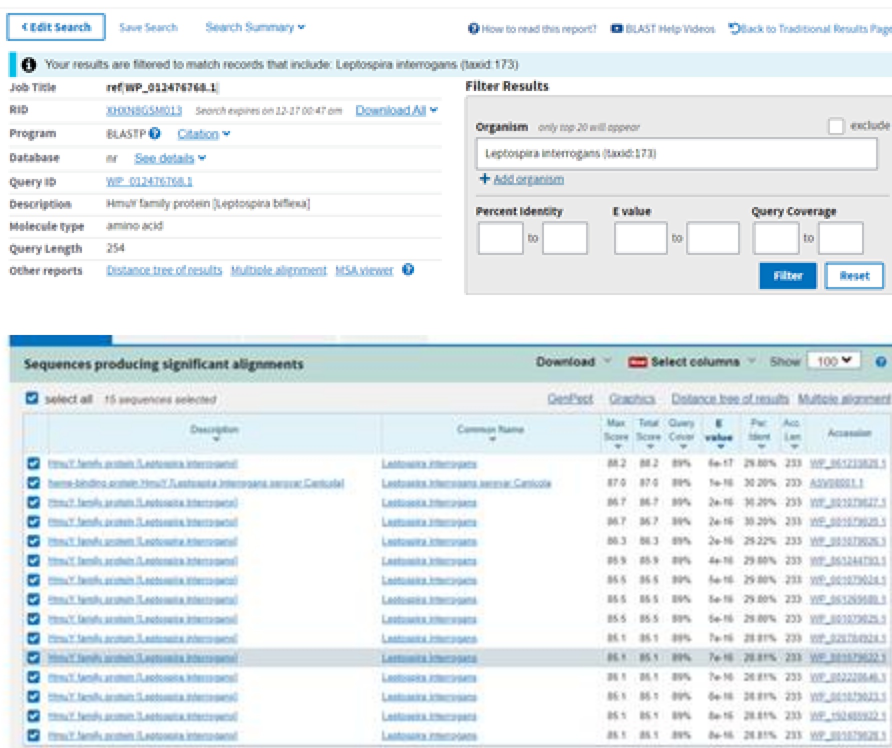

U.S. National Library of Medicine National Center for Biotechnology Information. (n.d.). Protein BLAST. Retrieved from https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome

Figure 9: This figure displays screenshots of the BLAST results for *L. biflexa*'s 254 amino acid HmuY family protein that include similarities to *L. interrogans.* The identity match to the HmuY-like heme-binding protein present in *L. interrogans* is about 30%.

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IV. Proposed Methods

In order to obtain experimental evidence that the HmuY-like heme-binding protein present in *Leptospira interrogans* does indeed play a vital role in the leptospire's survival and successful conquest of its host cell via successful binding of heme, the following experimental steps must be carried out. These must take place in a lab equipped to handle risk level 2 organisms. Before setting foot in a lab though, the gene sequence of the HmuY-like heme-binding protein must be analyzed. An online tool, such as ExPASy Translate© will be necessary to determine whether the gene can be translated and subsequently expressed in *E. coli*. If the sequence does indeed translate, then primers will need to be designed so that the gene can be cloned into a plasmid. An online tool, such as NEBcutter© can be used to determine which restriction enzymes should be used to cut the gene.

Next, cDNA should be ordered, and the previously designed primers should be used to amplify the gene via polymerase chain reaction (PCR). The PCR sample should be run on an agarose gel to separate the DNA from the chemical and enzyme components of the reaction. Once the gel is run, the DNA band that is produced should be cut out and the PCR product purified from the agarose. The DNA should be quantified using NanoDrop© and then, a restriction double digest should be set up employing the two restriction enzymes that were selected to cut the DNA. This will result in the ends of the amplified gene being cut off leaving 4-nucleotide overhangs, or "sticky ends". A separate double digest should be done on the empty plasmid in another tube resulting in a linear plasmid with corresponding sticky ends. The digests should be run on an agarose gel another time following this step, and the bands should be cut out and the digested PCR products and digested plasmid purified from the agarose. The digested amplified gene, or insert, and the digested plasmid should then also be quantified using

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NanoDrop©. A ligation reaction should then be prepared using a 3:1 insert to plasmid ratio and allowed to run overnight. Once the ligation reaction is completed, the plasmid now containing the gene for the HmuY-like heme-binding protein can be inserted into *E. coli* cells via transformation. DH5-α *E. coli* should be transformed with the newly created plasmid to create a mutant stock for long-term storage. BL21 *E. coli* should be transformed for subsequent protein expression studies. Once the cloning of the gene into the plasmid is complete, sequencing will be performed on successful transformants to ensure that the gene is in-frame and does not contain any replication errors or mutations.

The next portion of the experiment should be done to express HmuY-like heme-binding protein in transformed BL21 *E. coli.* A cell culture should be grown in 1 Liter of LB and ampicillin and incubated at 37°C until the culture reaches an OD_{600} of 0.4-0.6. Next, 0.5-1.0 µM allolactose-resembling IPTG should be added to the culture. The IPTG will cause the repressor to fall off the *lac* operon, inducing expression of the HmuY-like heme-binding protein cloned into the plasmid. The culture should be allowed to grow overnight at a cooler temperature, such as 18-21°C, as it produces ample amounts of protein steadily and with decreased formation of protein aggregates, or inclusion bodies, that could severely hamper total protein yield.

The protein will then need to be extracted and purified. The bacterial culture will be centrifuged at 12,000 RPM for 15-20 minutes in a Sorvall RC-5+ standing centrifuge. All the waste media should then be removed, and the pellet resuspended in 20-40 mL of ice-cold binding buffer. Cell lysis should then be performed using enzymatic lysis, mechanical lysis, or a combination of the two to ensure thorough lysis. The crude lysate should then be filtered through a 0.8-micron syringe filter, followed by a 0.45-micron filter, and possibly also a 0.22-micron filter using a syringe designed to hold greater than 30mL. The clarified lysate should then be

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passed through a column containing Ni+ coated agarose beads designed to purify His-tagged proteins, such as a GE Healthcase HisTrap column. After the lysate has been passed through the column, the column will be washed multiple times with binding buffer in order to wash and remove additional proteins leftover in the lysate. Elution buffer containing a high concentration of imidazole will then be used to elute purified HmuY-like heme-binding protein. A desalting procedure should be performed to complete the purifying process via either PD-10 desalting columns or a dialysis procedure.

Once the HmuY-like heme-binding protein is purified, the experimental options to determine the importance of the protein to *L. interrogans* and whether it is a virulence factor are endless. One could do a heme-binding assay in order to measure the heme-binding capabilities of this HmuY-like protein, run an SDS-PAGE and stain the resulting gel with Coomassie blue in order to determine the protein's size, and perform a protein crystallization experiment to characterize its structure. One could also culture wild-type *L. interrogans* and then grow them in various conditions and perform quantitative PCR to measure HmuY-like heme-binding protein gene expression, and infect various animal and human cell lines and again measure gene expression and protein production in the cells. CRISPR Cas9 methods could also be used to produce *L. interrogans* that lack the HmuY-like heme-binding protein. This would open the door to further experimentation that may include comparing growth characteristics of wild-type *L. interrogans* and the HmuYdeficient mutant, checking for gene expression in the wild-type vs. the mutant using housekeeping gene comparisons as a control, and performing *in vitro* infections of animal and human cell lines with both wild-type and HmuY-deficient mutant *L. interrogans* and recording differences in immune response or cell death. If the results of these experiments are significant, the next step would involve designing *in vivo* experiments, which will involve

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infecting an appropriate animal model, such as mice, with wild-type and HmuY-deficient *L. interrogans* and recording differences in immune response or cell death that may conclusively be attributed to the difference between the presence and absence of the HmuY-like heme-binding protein.

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V. Discussion

If the aforementioned experiments have positive results, meaning that the heme-binding assay reveals strong workings of an HmuY-like heme-binding protein, the crystallized protein has an uncanny structural resemblance to HmuY heme-binding proteins of other species, and it is conclusively shown that leptospires altered to lack this protein have a comparatively less effective establishment within their host cells, (among other inclining results), then it can be theorized that the HmuY-like heme-binding protein contributes significantly to the overall pathogenicity of *L. interrogans.* It will then likely be added to the current list of leptospiral virulence factors that could be a focus for therapeutic intervention. The HmuY-like heme-binding protein may even aid in solving the vaccination predicament that currently exists. If a vaccine could be developed to target this protein, we may no longer rely on vaccines that are effective only against certain strains of leptospira, as the HmuY-like heme-binding protein appears to be present in several major pathogenic leptospiral strains. Perhaps in contrast to the total current lack of research articles about this understudied protein, this work will one day inspire a newfound interest in it, resulting in papers containing novel insights into this possible leptospiral virulence factor's intricate functioning. On the other hand, if the previously outlined experiments produce negative results, then though we will all no doubt be disappointed, the point of this paper will not be moot. To the contrary, our work will still serve as a useful review article for those desiring to explore *L. interrogans* in all its wonder and will have paved the way for the investigation of others of its hypothetical proteins.

Indeed, *L. interrogans* has not historically been given the attention it deserves based on the rate of occurrence of Leptospirosis and the ugliness of its manifestation, both in humans and canines. As was previously mentioned, in both dogs and humans, mild leptospiral infection can

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cause severe headaches, stomachaches, muscle aches, chills, redness of the eyes, and jaundiced skin[2]. More severe infection can lead to myocarditis, pulmonary hemorrhage, liver failure, kidney failure, and even abortion of unborn fetuses in affected women[2]. It has been reported that more than 1 million humans are infected with Leptospirosis annually[55]. Of these, an estimated 58,900 infected individuals lose their life to *L. interrogans* every year[55]. As for canines, in 2014, Switzerland alone reported an annual peak incidence of 28.1 Leptospirosis infections per 100,000 dogs[27]. Thus, as long as our fellow humans and our beloved dogs are still suffering from ugly Leptospirosis infections, we owe it to them to continue researching the most common pathogenic source, *L. interrogans,* until we uncover all its biological secrets.

It is quite shocking to note the lack of published information regarding the virulence factors, mechanisms of infection, and host immune cell interactions of *L. interrogans.* One would think there would be a more complete collection of this important information, adequate to fill much more than a couple paragraphs per specified section. However, our search resulted in a limited number of details that left out a mapped, chronologically ordered, stepwise outline of Leptospirosis infection starting with *L. interrogans* entering the mucous membranes of the nose, mouth, and eyes, or through surface wounds on the skin[5] and ending with host cell apoptosis or recovery, depending on if and how *L. interrogans* was defeated and removed. We also were not provided with the same sort of detailed stepwise outline for the host cell's response to *L. interrogans* infection, but instead found piecemeal details explaining only certain aspects about the pathogen-host cell interaction. For example, we know that cytokines and chemokines, serum mediators, arginine vasopressin, soluble serum stimulation-2, long pentraxin, and nitric oxide are each involved in the host immune response to *L. interrogans* infection[41], but does this paint a clear and *comprehensive* picture of the host cell response during leptospiral infection? We think

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not. Furthermore, we find it very interesting that there are currently only twelve virulence factors that have been identified in *L. interrogans,* while there are still proteins that are classified as hypothetical, such as the HmuY-like heme-binding protein discussed here, which could very well play a role in the pathogenicity of *L. interrogans.*

We were further interested to note the lack of a detailed explanation of the structure of *L. interrogans.* Though it shares similarities to other spirochete pathogens, again we are not given a clear picture of the entirety of the leptospire's structure[11]. Rather, the literature places the vast majority of emphasis on the cell membrane and flagellar features of *L. interrogans*[11]. Since we do not even fully comprehend the structure of the most common agent of Leptospirosis, it is no surprise that we are lacking when it comes to its full capabilities during infection. *L. interrogans* has waged biological war against us humans and our beloved pet dogs for centuries and it has done so successfully, yet we still lack full or even adequate understanding of the means by which it does so. If our goal is to prevent the suffering and death caused by Leptospirosis, then research efforts like those we present here must be enacted and expanded now.

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Vita

Antonia M. Merta was born in San Antonio, Texas, on November 12, 1998 and raised in the country just outside of town. She attended elementary and middle school at St. Pius X Catholic School, and graduated 13th in her class from Antonian College Preparatory High School in May 2017. She began her college education at St. Mary's University during August of the same year and received the degree of Bachelor of Arts in Biology in May 2021. Her deep love for animals and learning motivated her to work with local veterinarians starting at age sixteen, and she aspires to become a Doctor of Veterinary Medicine herself one day.

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