CONSEQUENCES OF GENETIC DIVERSITY FOR HOST CELL ADHERENCE BY THE BACTERIUM *HELICOBACTER PYLORI*

by

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A THESIS

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Host and pathogen interactions like that of *Helicobacter pylori* and the human CEACAM1 N-domain are heavily reliant on specific amino acid interactions. Even a single change in a sequence can result in drastic changes in binding affinity for the interaction on either side. Understanding which amino acids create these significant bonds is key to developing treatments to common illnesses associated with *H. pylori* infection. CEACAM 1 interacts with *H. pylori* strains via one of two similar surface proteins called HopQ Type I and HopQ Type II. These proteins share a 70% sequence homology in the binding domain yet their relative binding affinities to *H. pylori* strains are relatively unknown. The goal of this study is to compare binding affinities of these two proteins with a variety of CEACAM 1 variants across humans and primates in order to identify the sequences that lead to these significant alterations in binding affinity.

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Introduction

The human body is full of resources which allow all our cells to continue functioning every second of our lives. Some of these resources are nutrients such as sugars or carbohydrates which are floating around in our blood while others, like the iron in our red blood cells, are locked away behind cell walls. Other resources include certain cellular machinery which allow for general function and replication. Although this plethora of resources is important for our survival, it is not without its consequences. Many pathogens require these resources for their own survival and replication, which makes humans a habitable environment for many pathogens.

To prevent colonization by pathogens, humans have evolved defensive barriers throughout the body. This includes cellular membranes, mucosal linings, immune system proteins and diverse pH environments. However, diseases caused by pathogen colonization still occur, as certain pathogens have acquired the ability to move around these protective measures. One common battleground which either prevents or allows invasion from a pathogen is the cell wall. Pathogens have found a number of ways to bypass the cell wall including some that use toxins such as pore-forming toxins(PFTs) to break open the lipid bilayer that makes up the cell wall(Gonzalez et al., 2008). Opening the cell wall allows the vital nutrients locked within a cell to flow into the extracellular space for pathogen consumption. Another common technique used by pathogens to infiltrate cellular membranes is the use of proteins to fuse with the lipid bilayer thus allowing the insertion of pathogen DNA, RNA, or proteins(Arias & Dubois, 2017). A final approach that pathogens have developed to neglect our cell's protective layer involves binding with and altering surface proteins. These proteins are

imbedded in the lipid bilayer of a cell and thus can serve as an access point into the intracellular region. Cells with surface proteins that are unable to prevent invasion are infected and allow the successful pathogen strain to proliferate using the nutrients and cellular machinery housed inside the cell. If this pathogen-favored interaction occurs frequently enough this could weaken or kill the host. This decrease in fitness for the host due to this protein which allows pathogen binding could lead to selection against this specific protein in the population. Contrary to this outcome, if the surface protein prevents invasion, then the pathogen is unable to acquire nutrients and will likely die. Like in host proteins this could cause this pathogen protein to be selected against in the population in favor of a slightly different protein which allows binding to occur. This type of interaction results in a situation where on a population scale both human surface proteins and pathogen adhesion proteins experience rapid coadaptation. This rapid change in proteins is due to the continual shift of the dominant genotype of human surface proteins and pathogen strains as previous genotypes are selected against by successful competitors. This evolutionary cycle based on biotic interactions was first described as the Red Queen Hypothesis (VAN & L, 1973). This hypothesis describes how biotic factors can drive a form of zero sum evolution where species are constantly adapting to maintain an average level of fitness relative to their competitors(Figure 1).

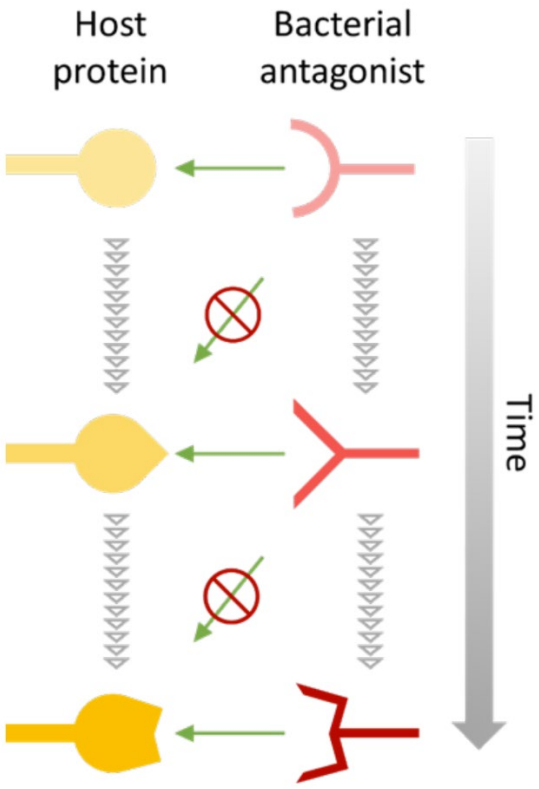

Figure 1: Host-pathogen coadaptation due to chronic interactions (Baker et al., Unpublished)

Host-pathogen interactions can result in continual coadaptation from both proteins. In this instance binding is advantageous for the bacterial antagonist which drives a shift in the host protein. A lack of binding is advantageous for the host protein and drives a shift in the bacterial antagonist. This results in a roughly net-zero relative fitness gain for both species.

The factor that primarily determines whether invasion of a pathogen occurs, when interacting with a surface protein, is the ability of that pathogen's adhesion protein to bind to the surface protein. This binding is orchestrated by hydrophobic or hydrophilic amino acid interactions, disulfide bonds, hydrogen bonding, and many other types of interactions. The binding affinity of these highly sensitive interactions can be significantly altered by even a single amino acid change caused by a nonsynonymous point mutation in the genotype. When a species or strain has a ratio of nonsynonymous

to synonymous mutations greater than one, this can indicate that positive selection is occurring. Positive selection is the rise of an advantageous mutation in the genotype of a population due to selection factors creating an environment where that mutation significantly increases fitness of its host.

Although rapid nonsynonymous changes in human surface proteins allow for a reduction in the binding affinity of pathogen proteins these receptors typically exist to interact with other host proteins to perform vital functions. These host-host interactions are just as sensitive as host-pathogen interactions, so the nonsynonymous changes that prevent invasion can also result in a loss of function for that protein.

Overall, host-pathogen interactions are a contributing force to shaping the genotypes of human populations across the world. Identifying host-pathogen interactions, and the consequences resulting from this interaction is an important area of study that can shed light on how these interactions can be prevented. Within this field of study, an example of particular interest involves human CEACAM proteins. CEACAM (CarcinoEmbryonic Antigen-related Cell Adhesin Molecules) are a family of proteins involved in cell-cell recognition and several cellular processes including T-cell proliferation and insulin homeostasis(Figure 2:Kuespert et al., 2006). It was observed that many of these proteins were experiencing positive selection in their N-domain which is a common binding spot for other host proteins and pathogens(Adrian et al., 2019; Voges et al., 2010).

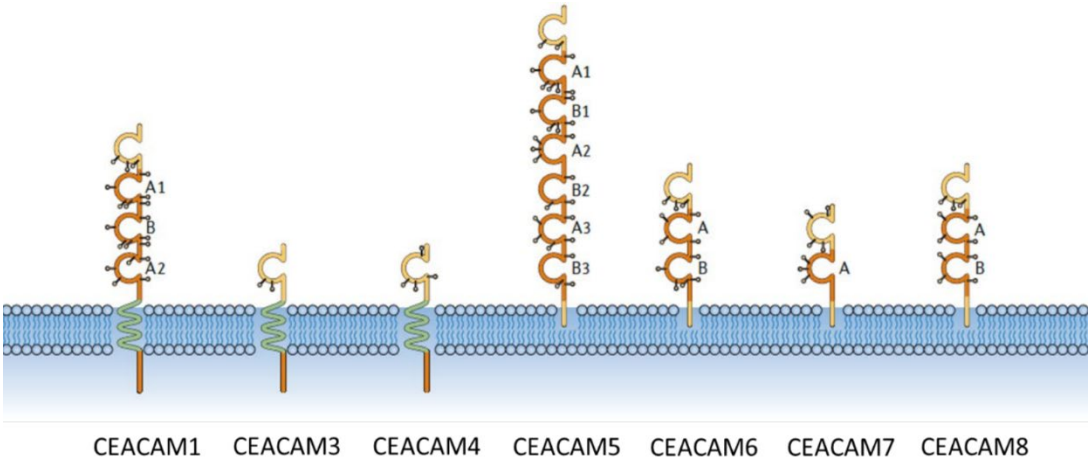

Figure 2: CEACAM family proteins (Adapted from Grey-Owen and Bloomberg, 2006)

Extracellular CEACAM proteins all contain a N-terminus protein(Yellow) which is a frequent binding spot for pathogen and host proteins. Many proteins have additional similar extracellular units(orange) and a transmembrane domain(green).

One bacterium that is likely contributing to this positive selection in CEACAM proteins is *Helicobacter pylori(H.pylori)*. This bacterium is currently estimated to colonize the stomach of 50% of the world's population and has been found to have been coevolving with humans for 50,000 years(Atherton & Blaser, 2009). *H. pylori* utilizes an adhesion molecule called HopQ to bind with a variety of host CEACAM proteins(Bonsor et al., 2018). Two families of genetically and geographically distinct HopQ have been identified and are commonly referred to as Type I and Type II. On average, Type I and Type II strains share 70% amino acid homology in the binding domain, and Type I is typically found in the Middle East and Asia while Type II is typically found in the United States and Canada(Cao et al., 2005). A major distinction between the two strains of particular importance to the medical community is the presence of the Cag pathogenicity island or group of genes almost exclusively found in Type I genomes(Cao et al., 2005). Binding of HopQ to CEACAMs can lead to the

implantation of a protein called CagA if the strain has the group of Cag genes. This protein has been linked to a large increase in the risk of gastric cancer (Hatakeyama, 2014). Some estimates suggest that this type of interactions with *H. pylori* causes one million annual cases of gastric cancer worldwide(Wroblewski et al., 2010). This resulting increased risk of gastric cancer lead the World Health Organization to label *H. pylori* the first bacterial carcinogen in 1994(Parkin et al., 2005).

From a host-pathogen perspective this increased gastric cancer risk from *H.pylori* could act as a selection factor for individuals with CEACAM proteins that bind with HopQ. As there is positive selection on many CEACAM protein N-domains, the question became which CEACAMs are being bound by HopQ? It has been shown that both HopQ types will bind to CEACAM1, 3, 4, and 6(Javaheri et al., 2016). This same study reported that CagA insertion only occurred when HopQ attached to CEACAM 1 which suggests *H. pylori* could be partially responsible for the rapid evolution in the N-domain of human CEACAM1(Javaheri et al., 2016). It should be noted that CEACAM3, which aids granulocytes in recognizing and consuming bacteria that bind to other CEACAM proteins, could also be changing due to HopQ binding(Adrian et al., 2019). Individuals with CEACAM 3 proteins that are not able to identify HopQ would not have the opportunity to stop an infection from occurring.

When examining HopQ binding with the CEACAM1 N-domain the main area of binding seems to be in the C1ND region in CEACAM1 which binds with both isoforms of HopQ: Type I and Type II (Figure 3)(Moonens et al., 2018). Despite this binding interaction being well documented the specific amino acids that are essential for binding have not yet been identified. In addition, *H. pylori* is primarily found only to

inhabit humans and higher primates; no published work has been done to compare binding affinity between HopQ types and different types of CEACAM1 proteins across higher primates and humans (Atherton & Blaser, 2009). If *H. pylori* strains that colonize humans were observed to bind to primate CEACAMs, this could demonstrate whether human specific *H. Pylori* would cause issues if transferred to primates, and it could also allow us to compare N-terminal amino acid sequences between binding and non-binding CEACAM1s to locate important binding amino acids. To obtain this data the Barber lab has already examined binding of HopQ type I across several primate species' CEACAM1s. They found that human HopQ type I strains G27 and J99 bound to CEACAM1s from chimps and gorilla (Baker, Unpublished data). Interestingly, they also found that bonobo CEACAM1 did not bind either type I strains while gorilla CEACAM1 bound with both strains. This is unexpected as bonobos are phylogenetically closer to humans than gorillas, which would suggest that bonobos are less likely to have diverged enough genetically to lose binding compared to gorillas. To examine whether the same CEACAM1 proteins show similar binding to the genetically distinct type II HopQ strains western blotting was done with the same primates and human HopQ type II strain Tx30a. This could allow comparison of the strains to determine important amino acids for binding.

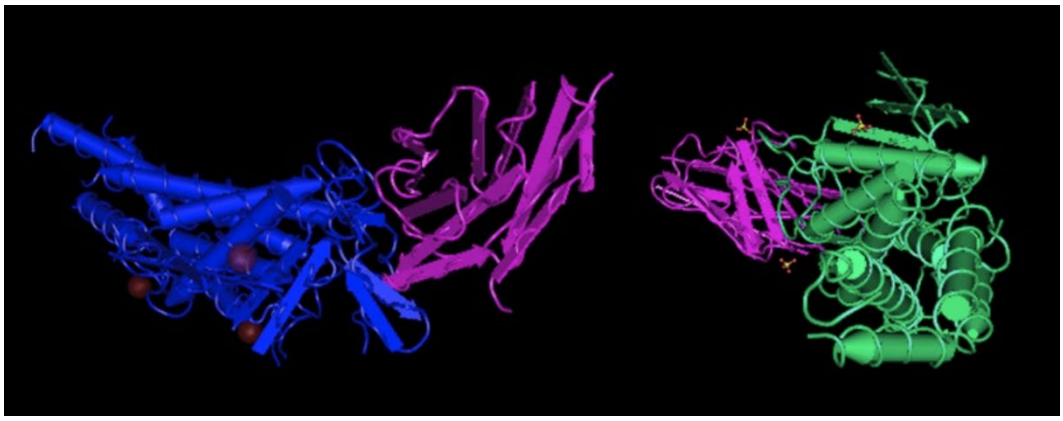

Figure 3: Interaction of HopQ type I and Type II with C1ND region of CEACAM1 N-terminus (Adapted from Moonens et al,. 2018)

The *H.Pylori* alleles HopQ type I(Blue) and HopQ type II(Green) have a 70% homology in their binding region which translates to unique interactions with the C1ND region of the CEACAM1 N-terminus(Purple).

To further explore why binding with CEACAM1 in bonobos was lost the Barber Lab created human and bonobo CEACAM1 mutants that switched amino acids which differed between the two proteins in areas of human CEACAM1 that were found to be experiencing positive selection(Figure 4). The mutants created are labeled humG85Q, bonQ85G, humNHI(61-63), and bonQLF(61-63) with lettering on the left noting the wild type amino acid replaced by amino acid letter noted on the right. The number represents the amino acid distance from the N-terminus. These mutants were paired with G27, J99 and Tx30a strains to observe if binding would occur. A recovery of binding in any bonobo mutants or a loss of binding in human mutants would strongly indicate that the switched amino acid was necessary for binding or responsible for lack of binding.

A study done in 2019 identified 3 CEACAM1 mutants that interestingly were commonly found as a group and not independent, and comprised roughly 10% of certain African populations(Adrian et al., 2019). These mutants are Q1K, A49V, and

Q89H which are numbered from the N-terminus of the CEACAM1 binding area. Although not directly associated with the primate panels these mutants could also offer insight into valuable binding sites for HopQ. These mutants were tested with G27, J99 and Tx30a independently and also grouped together.

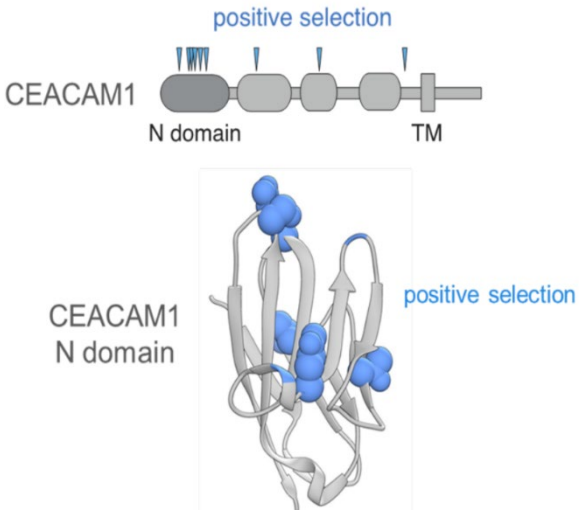

Figure 4: Areas of positive selection found on human CEACAM1 N-domain (Adapted from Emily Baker)

A 3-D representation of human CEACAM1 N-domain with sites found to be experiencing positive selection(blue). Although positive selection was found across CEACAM1 it is heavily concentrated in the N-domain which is a common site for binding. TM represents the transmembrane portion of CEACAM1.

Methods

To examine the qualitative ability of *H. pylori* to bind to primate CEACAM1 proteins, human/bonobo CEACAM1 mutants, and CEACAM1 African mutants each of these respective protein's N domain was tagged with a green fluorescent protein (GFP) for optimal visualization. After these proteins were expressed in mammalian cells, they were incubated with specific *H. pylori* strains in a pulldown assay. This assay allows for the interaction of two or more proteins for the eventual observation of binding if such affinity exists. After the interaction period these samples were washed and suspended in a laemmli buffer which caused the proteins to denature. The denatured proteins were then used in a western blot, a procedure which uses an electrical signal to pass samples through a gel to determine the relative size of DNA, RNA or protein samples based on distance traveled. After size determination the gels are transferred to a nitrocellulose membrane which can be used to administer antibody treatments. These treatments involved a primary antibody, which bound to the GFP attached to the CEACAM1 N domain proteins. After removing any unbound primary antibody via a wash, a secondary antibody that attached to the primary antibody was applied. This secondary antibody contained an enzyme that can produce visible light via chemiluminescence which could then be imaged using charge-coupled device digital imaging.

CEACAM Protein Prep

Primate CEACAM1 expression plasmids were transfected into Human HEK293T cells using the Lipofectamine™ 3000 transfection kit from Invitrogen following manufacturer's instructions. Two days after transfection cell supernatant was

collected and filter sterilized, and cells were collected and lysed. Expression of proteins was confirmed by western blotting.

Bacterial strain growth conditions

H. pylori type I strains G27 and J99 and type II strain Tx30a were plated onto horse blood agar plates, from glycerol stocks kept at -80º, which were then placed under aerobic conditions (10% CO_2 at 37º) for 96 hours. Colonies were then removed from the agar and added to a brain-heart infusion broth prior to combination with CEACAM protein.

Protein pulldown

H. pylori cultures suspended in a brain-heart infusion broth were combined with GFP-tagged CEACAM 1 N-terminal domain proteins from varying primates at room temperature for 30 minutes while being rotated. The samples were then put through two cycles of a 1x PBS wash followed by being spun down in a centrifuge at 10000rpms for five minutes. A 20 to 1 mixture of BIO-RAD 1x laemmli sample buffer with 2-mercaptoethanol was added to samples that were then boiled at 95º for five minutes.

Western blot

20µL samples of CEACAM1 protein with *H. pylori* bacterial cultures were loaded into Mini-PROTEAN TGX gels before electrophoresis. Separated proteins were then transferred to a nitrocellulose membrane. These membranes were incubated in 5% non-fat milk infused with primary antibody (monoclonal, anti-GFP mouse) for one hour(Figure 5). This was followed by three 1x PBS washes before incubation with the secondary antibody (monoclonal, HRP-conjugated anti-mouse goat) and the ladder

antibody (HRP- conjugated precision protein strep-tactin) for twenty minutes. The membranes were then washed with 1x PBS again before imaged using a chemiluminescent western blot imager (LI-COR Biosciences).

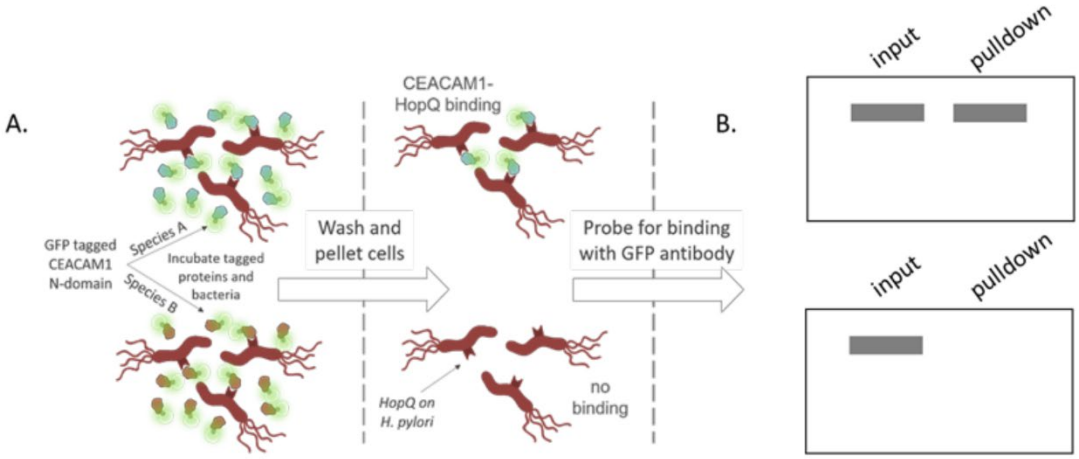

Figure 5: Protein pulldown assay and western blotting procedure (adapted from Emily Baker)

Various primate GFP-tagged CEACAM1 N-domains were incubated with individual strains of *H. pylori*. These were then washed with 1x PBS and spun down with a centrifuge. Binding was verified by soaking in 5% low fat milk with a GFP antibody derived from mice. This was followed by a secondary mouse antibody derived from goats. Input lanes contained pure GFP-tagged CEACAM1 N-domain while pulldown lanes contained the GFP-tagged CEACAM1 N-domain incubated with *H. pylori*.

Results

Western blots of the primate panel including *H. pylori* Type I strains G27 and J99 along with Type II strain Tx30a revealed similar binding between all three strains.

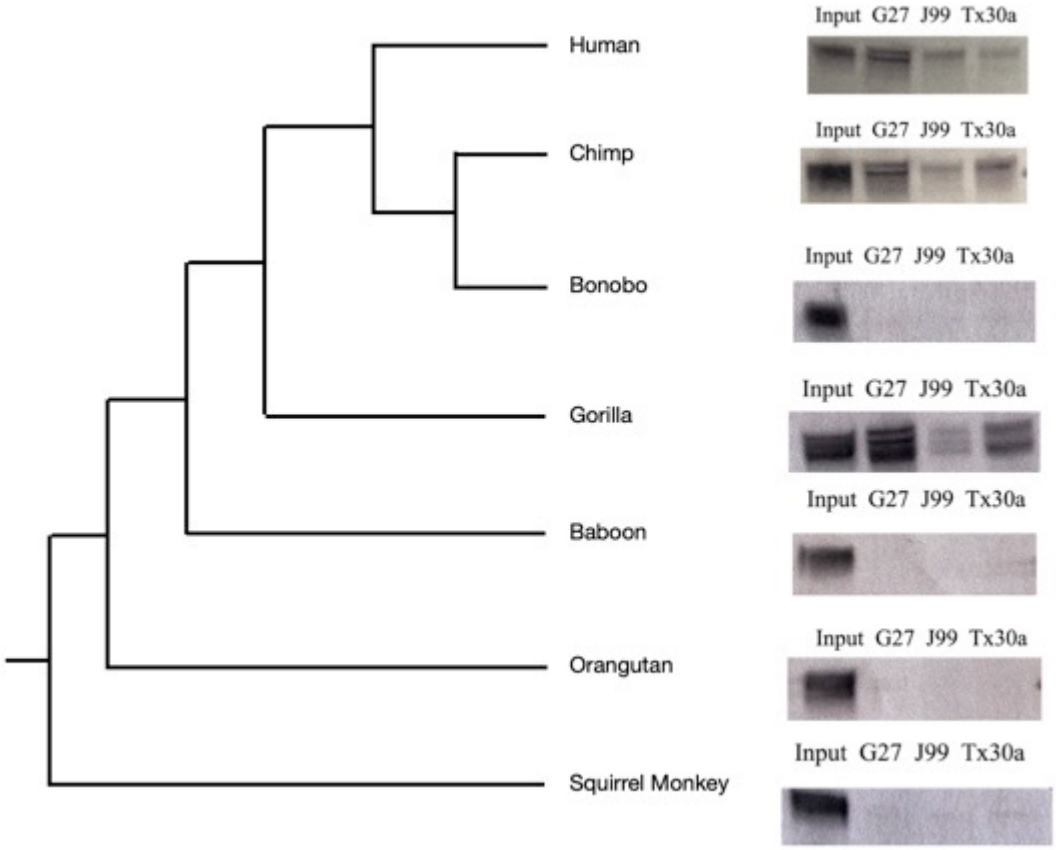

Figure 6: Primate panel western blot data with *H. pylori* strains G27, J99, and Tx30a

Primate CEACAM1 N-domain binding with HopQ type I strains G27 and J99 along with HopQ type II strain Tx30a showed that chimp and gorilla CEACAM1 N-domain bound all *H. pylori* strains. The lack of binding to bonobo CEACAM1 N-domain appears to be present in both types of HopQ despite a closer common ancestor than gorilla. Varying binding affinity should be carefully inferred from binding results.

Western blots of the avaliable human and bonobo mutants showed no binding when human CEACAM1 swapped certain amino acids with bonobo CEACAM1 when

binding Tx30a. This was not matched by a gain of binding when bonobo CEACAM1 swapped certain amino acids with human CEACAM1. Western blots of various combinations of the 3 common CEACAM1 N-domain mutants in certain African populations showed continued binding with Tx30a.

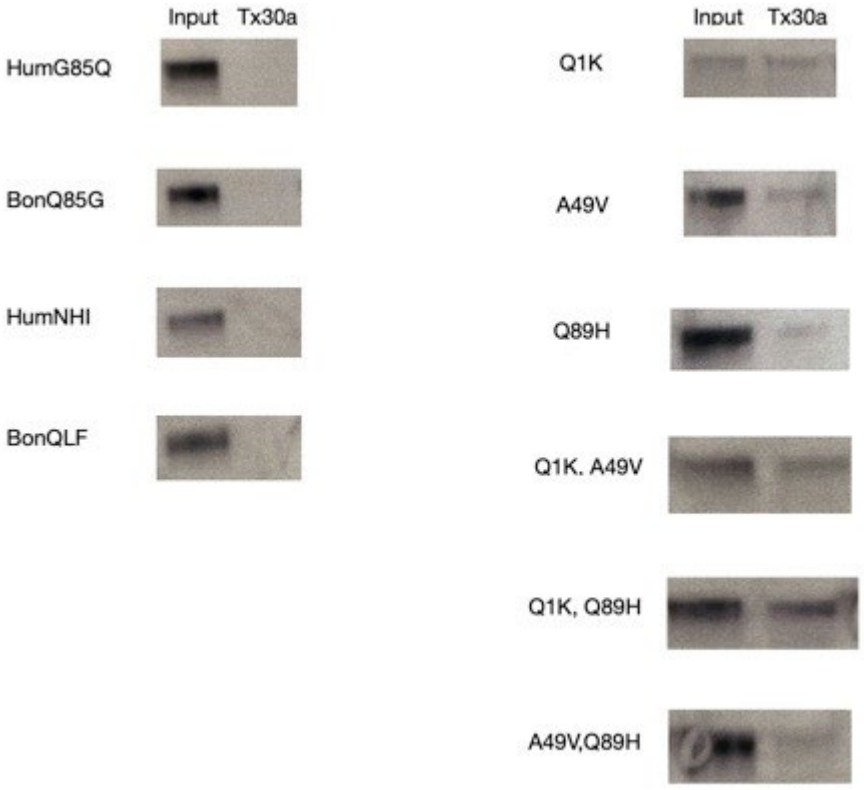

Figure 7: Western blots of human/bonobo CEACAM1 mutant, and 3 African CEACAM1 mutants

A loss of binding was observed with the human CEACAM1 proteins with bonobo amino acid inserts. Binding was not restored when bonobo CEACAM1 proteins had human amino acid inserts. Despite varying levels of prominence, all African CEACAM1 mutants bound Tx30a. Varying binding affinity should be carefully inferred from binding results.

Discussion

Observing that the HopQ type II strain Tx30a bound to the same primate CEACAM1 proteins as the Type II strains G27 and J99 is both exciting and surprising. The similar binding of type I and Type II HopQ strains despite only a 70% sequence homology suggests that both types have achieved unique, but similarly effective methods of binding. It should be noted that the nature of evidence from western blotting is qualitative so binding strength can only be estimated. It would be greatly informative in the future to use flow cytometry to obtain more quantitative binding affinities for comparison between primate CEACAM1 proteins. Flow cytometry measures the density of cell populations using light, and can also be used to detect binding of proteins if a control template is used for comparison. The lack of binding to orangutan, baboon, and squirrel monkey CEACAM1 is expected as these are phylogenetically distant from humans. The lack of binding to baboon that was already observed in type I strains G27 and J99 is intriguing and deepens the curiosity surrounding what is causing rapid non-synonymous mutations of CEACAM1 in bonobo populations.

Although the data on bonobo mutants is preliminary and only includes part of Tx30a, if the data is correct, it could be very informative. The loss of binding in both humG85Q and humNHI shows that the original human amino acids are necessary for binding to occur. When this is paired with the lack of binding restoration in the bonQ85G and bonQLF this shows that the human amino acids in these positions are not sufficient for binding. This suggests that HopQ type II binding with human and bonobo CEACAM1 is supported by complex binding interactions from multiple points within the host protein and pathogen adhesion protein.

The CEACAM1 mutant data including Tx30a is a truly interesting start to the process. Despite the lack of information from G27 and J99 the fact that HopQ type II binds with all mutants and mutant combinations is suggestive that other factors might be the cause of the evolution of these CEACAM1 mutants. If binding to HopQ still occurs this would suggest that the likely advantageous nature of these mutations, which presumably caused its emergence in the population, would be due to altering a different binding interaction. If no change in binding with HopQ has occurred, then no change in fitness would drive these mutations to become more prevalent in a population. This is purely speculative and could also be because the mutations occurred and provided no disadvantages and so remained in the population at lower levels. This would not explain why the mutants are commonly found to be paired instead of independent which suggest that some advantage was derived from these mutations the nature of which being unknown. It must also be mentioned again that this data is qualitative in nature and does not definitively show if subtler binding affinity changes are occurring. A lowering of binding affinity due to the mutations could provide some advantage and would allow *H.pylori* to act as a selective force on these CEACAM mutants.

Future Directions

The obvious next step in this process is to continue the HopQ type I and type II comparisons with the human and bonobo mutations and the African population mutants. This will allow us to completely compare the two types of strains and what amino acids could be leading to any differences in binding. It could also allow us to observe if other amino acids that vary between human and bonobo CEACAM1 follow the pattern already seen with loss of function with the addition of bonobo amino acids to human CEACAM1, but no revival of binding with human amino acids added to bonobo CEACAM1. Continuing the examination of the African mutants with HopQ type I could show a difference in binding which suggests successful avoidance of HopQ type I strains by these mutants. If no difference in binding is found it would be important to examine whether other CEACAM protein such as CEACAM3 and CEACAM5, which also have been found to have common mutations, have different binding affinities to HopQ(Adrian et al., 2019).

As the *H. pylori* strains used in this are only a small fraction of all identified variants it would be beneficial in the future to perform these experiments with additional strains of both HopQ types already in use for research of HopQ and CEACAM1 binding. Strains already used in previous studies include type I strain P12 and type II strain 60190. Using strains already within the current literature will allow for comparability of our results as the complexity of this host-pathogen interaction requires a collaborative approach to understand.

A final recommended area of expansion for the experiment would be to find instances of positive selection occurring in HopQ type II. A comparison could then be

done to the sites observed on HopQ type I by the Barber Lab. This can be followed by mutating the type II sites to see how this impacts binding with CEACAM1.

Overall, this project has helped start the process of identifying amino acids that are important for the binding interaction of HopQ and CEACAM1. The hope for future projects would be to use this work to further understand the complex interaction of these two proteins. The eventual goal being the prevention of cagA insertion into human cells which could result in a significant reduction in *H. pylori* related gastric cancer cases worldwide. It was an honor to work on this project, and I am excited to see what future researchers can uncover.

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