

**CO-INFECTION STUDIES ON HEPATITIS C VIRUS AND MALARIA PARASITE
LIVER STAGES**

by

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Malaria and hepatitis C are infectious diseases that affect millions of people worldwide. These two diseases are caused by two different pathogens, *Plasmodium* parasite for malaria and hepatitis C virus (HCV) for hepatitis C, that share some similarities in their development within the hepatocytes of the liver. Co-infection of these two pathogens has largely remained unstudied, but due to epidemiological overlap, it is plausible that individuals can be afflicted with both malaria and hepatitis C. To date, it has been shown that *Plasmodium* parasites and HCV utilize four common host entry factors to gain entry into hepatocytes: Heparan sulfate proteoglycans (HSPGs), scavenger receptor-B1 (SR-B1), cluster of differentiation 81 (CD-81), and apolipoprotein E (apoE). ApoE incorporated into new HCV virions plays a key role in viral infectivity. In its entirety, our hypothesis states that given the increasing prevalence of hepatitis C in parts of the world where malaria is endemic, hepatitis C virus (HCV) and *Plasmodium spp.* Co-infections are a likely occurrence. In this case, it is plausible that co-infections with these pathogens will affect the replication of either pathogen during their liver stages. Furthermore, it is likely that *Plasmodium* parasites utilize claudin-1, occludin, and apoE host entry factors, which are important for HCV entry and ability to invade hepatocytes.

Using an *in vitro* model of infection in liver derived HuH7 hepatoma cells, we hope to look at the overall affects these pathogens have on one another through co-infection studies of *P. berghei* and HCV both together and individually. Furthermore, we hope to examine other

host factors that HCV utilizes for entry into hepatocytes and their affect on *Plasmodium* entry during the liver stages of infection. This study is significant to public health to improve existing anti-malarial and hepatitis C treatments by intervening at the early stages of each pathogen's development. By understanding how a pathogen enters, invades, and develops within a host, it is better understood how therapeutic drugs can target and decrease pathogenic development.

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PREFACE

This journey has been long with bumps and turns; however, with the help, love and support of certain individuals, my goal fell into place easier than I believed could be possible.

To Dr. Tarun, thank you for giving me the opportunity to work beside you. I have learned so much the past couple years and it has all been because of your guidance and knowledge. Dr. Wang, thank you for your understanding nature and answering all my questions.

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1.0 INTRODUCTION

While there are many epidemiological studies that examine the co-infection of malaria with viral diseases, such as HIV and hepatitis B, there is a lack of research focusing on the co-infection of malaria with hepatitis C virus (HCV).

Approximately 3% of the world's 170 million people have been infected with HCV [1]. Even though the prevalence of HCV is less than 3%, there are areas of the world where the prevalence is higher—15% in some countries in Africa, Egypt retaining a prevalence of higher than 15%, and Southeast Asian countries, which include India (1.5%), Malaysia (2.3%) and the Philippines (2.3%) as shown in Figure 1 [1, 2, 3, 4].

The standard treatment of ribavirin and interferon- α are becoming less effective due to increasing drug resistance in many of the different strains of hepatitis C; furthermore, a new anti-malarial drug is needed due to the resistance of *P. falciparum* to chloroquine, pyrimethamine, cycloguanil, sulfadoxine, atovaquone and other anti-malarial drugs [5,6]. Therefore, comprehending how both HCV and *Plasmodium* infect and are affected during co-infection in host hepatocytes will help interpret the pathogenesis of these pathogens. In turn, may lead to new insights into therapeutic measures for treatment.

Like hepatitis C, malaria is endemic in Southeast Asian countries as well as sub-Saharan African regions [7]. In fact, 90% of worldwide malaria cases infecting at least 500 million people are located in the sub-Saharan Africa, deeming it the most common parasitic disease in

the tropics, shown in Figure 2 [7,8,9]. Given the increasing prevalence of HCV in these parts of the world where malaria is endemic, co-infections could be a likely occurrence both for HCV and *Plasmodium spp.* In this case, it is also plausible that co-infection may affect the severity of either disease.

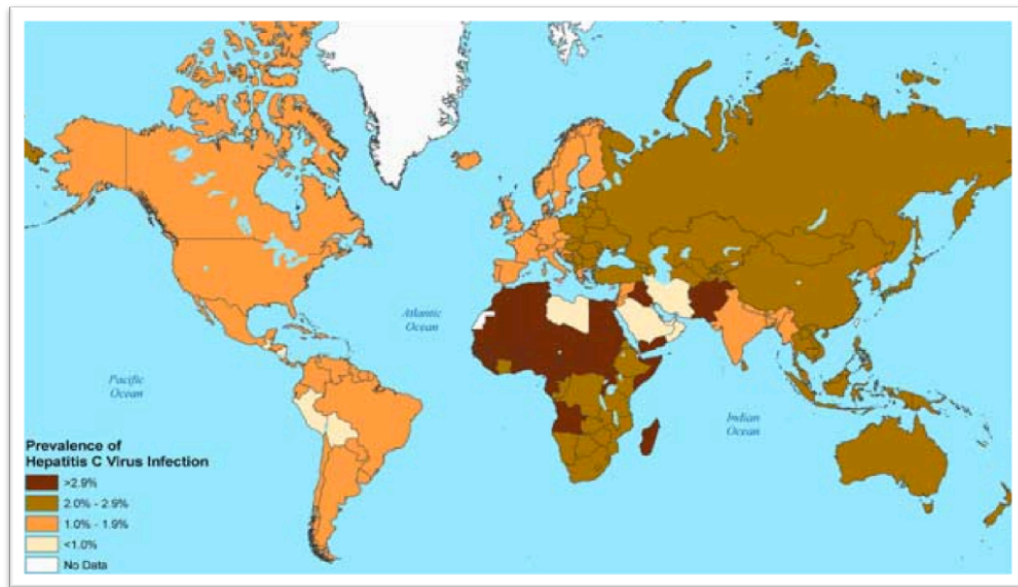


Figure 1. Estimated global prevalence of Hepatitis C virus infection [2]

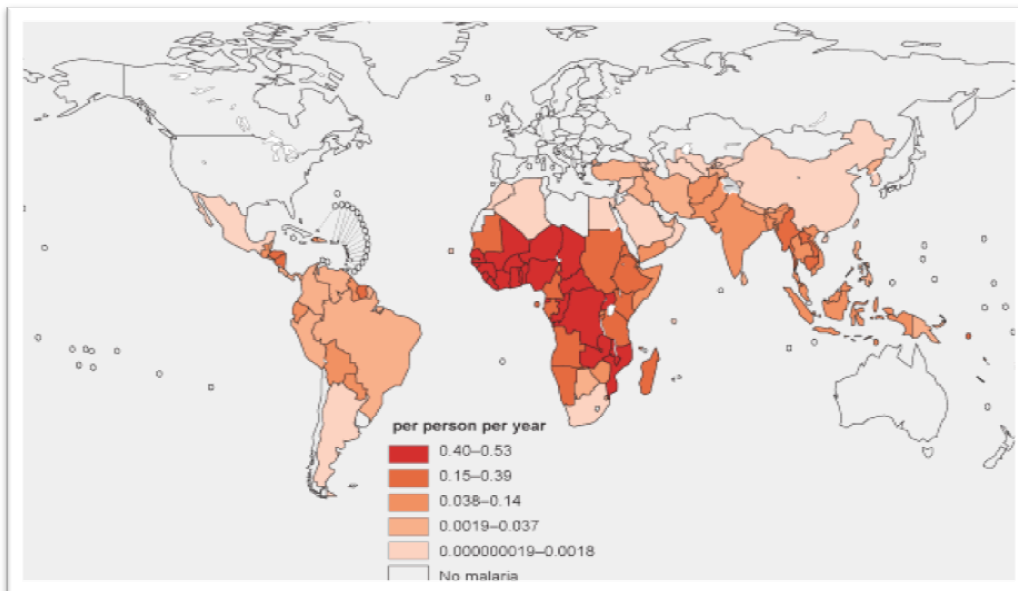


Figure 2. Estimated global incidence rate of malaria transmission [9]

1.1 HEPATITIS C

A chronic disease, hepatitis C affects 130-170 million people worldwide [1]. This disease is the leading cause of liver cirrhosis and hepatocellular carcinomas. Liver cirrhosis is defined as the deterioration and malfunction of the liver where scar tissue replaces healthy tissue in the liver causing loss of the ability to fight infections, filter blood, produce bile, and process nutrients, hormones and drugs; whereas, hepatocellular carcinoma is a cancer caused by complications due to liver cirrhosis [10,11]. Currently, entry of HCV into host hepatocytes has been studied heavily.

1.1.1 Hepatitis C Virus

A member of the family Flaviviridae, HCV is the pathogen responsible for causing the disease hepatitis C. Upon infection with HCV, the virus migrates through the blood stream to the liver where it enters the host's hepatocytes. The virus is composed of a positive single-strand of RNA, enabling the host cells to immediately translate the viral RNA. The 10kb viral genome encodes a polyprotein, which is processed by host and viral proteases [12]. The structural region of the polyprotein includes viral envelope glycoproteins E1 and E2, which are initially utilized for the overall binding and entry of the virus to host hepatocytes [13].

1.1.2 Hepatitis C virus entry and replication

Upon entry, the HCV E2 glycoprotein originally binds to heparan sulfate proteoglycans (HSPGs) via apolipoprotein association on the hepatocytes surface [14]. Subsequently, the virus interacts

with scavenger receptor B1 (SR-B1) and Cluster of Differentiation 81 (CD-81) post-binding the HSPGs [15,16,17]. Late claudin-1 and occludin co-receptor binding is required to enter the host cell [18]. These details regarding HCV entry are illustrated in Figure 3, which was modified based on Lanford, et al (2009) [18].

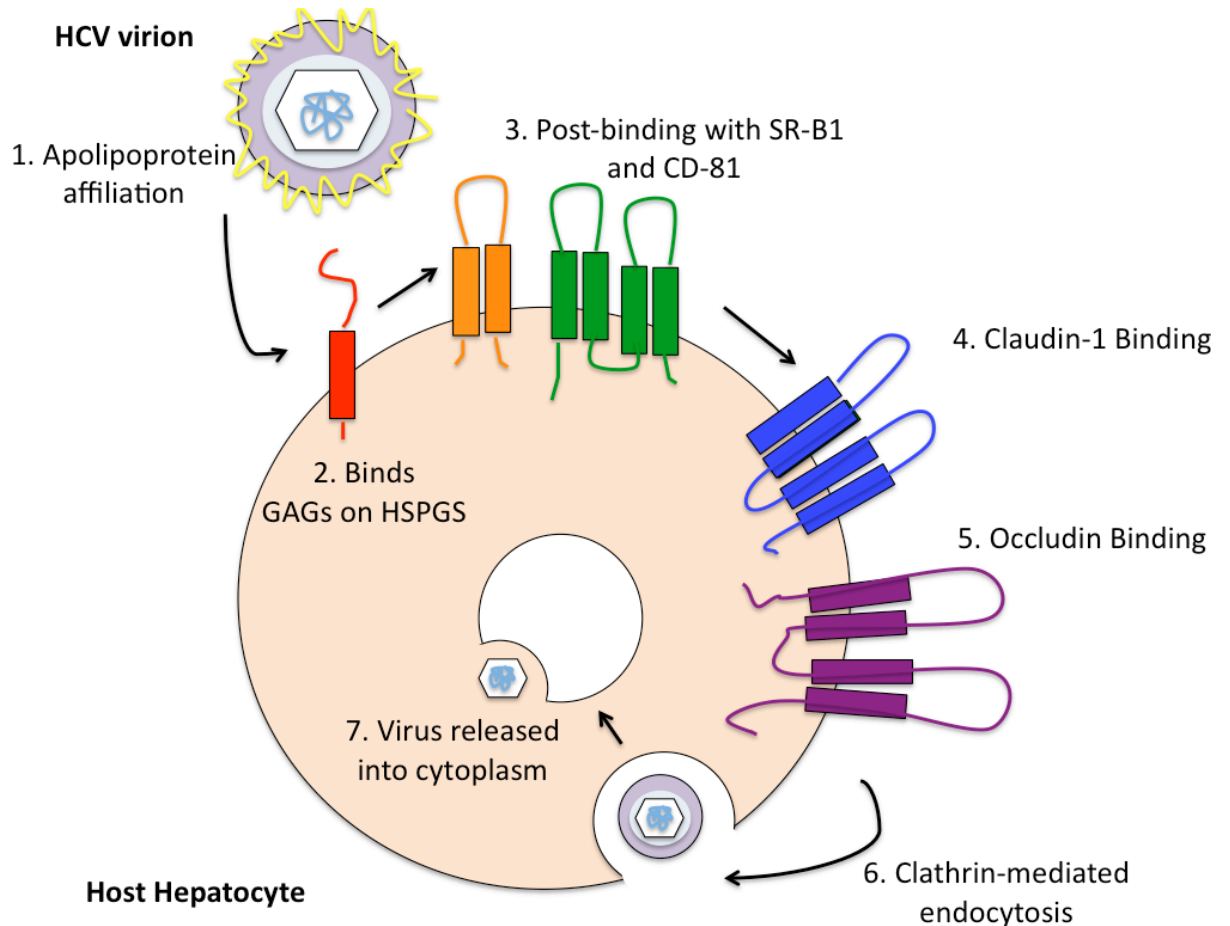


Figure 3. Hepatitis C virus attachment and entry into a host hepatocytes

Thereafter, the hepatocyte undergoes clathrin-mediated endocytosis, creating an endosome of viral entry [18]; this fusion of the virus to the endosomal membrane is low pH-dependent. At this point, the virus is uncoated and releases its positive single-stranded RNA into the host cells to be translated and replicated by host cell machinery. Virions are then assembled

and transported to the cells membranes where they are released from the host cell to infect other nearby hepatocytes as shown in Figure 4 [19,20].

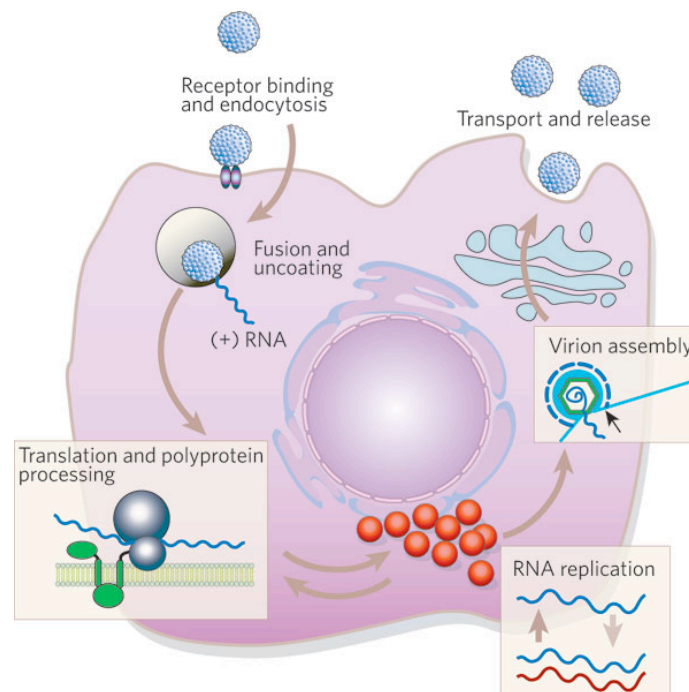


Figure 4. Hepatitis C virus life cycle in a host hepatocytes [19]

1.2 MALARIA

Like hepatitis C, malaria is also a major concern in many countries around the world, particularly in developing countries. Malaria is endemic in 105 countries and responsible for over 300 to 500 million clinical cases and more than a million deaths each year in developing countries [18]. Due to their lack of or compromised immunity, children, pregnant women and those with compromised immune systems are most at risk for malarial transmission [19]. In fact, in 2008, malaria caused approximately one million deaths primarily among African children [19].

1.2.1 *Plasmodium spp.*

The pathogen itself, *Plasmodium*, is a parasite and is transmitted by one of 50 to 60 different species of the Anopheles mosquito [20]. There are five known species of the parasite that causes malaria in humans, of which *Plasmodium falciparum* is the most infectious and lethal to humans, while *Plasmodium vivax* is the most widespread species. Other species include *Plasmodium ovale*, *Plasmodium malariae* and *Plasmodium knowlesi*. *P. knowlesi* is a primate parasite that is primarily found in Southeast Asia; it has been known to cause malaria in long-tailed macaques as well as in humans, making it one of the five known species of *Plasmodium* to infect humans.

1.2.2 *Plasmodium spp. Life Cycle*

The life cycle of the malaria parasite, shown in Figure 5, begins as sporozoites are released from the salivary glands of the Anopheles mosquito into the host's blood stream (Fig 5A). The parasites then migrate to the liver to begin the exoerythrocytic stage of the *plasmodium* life cycle (Fig 5B). The sporozoites enter the liver cells via HSPGs, CD-81, SR-B1, and other similar receptors shared by HCV (Fig 5B) [22,23,24]. Once in the hepatocyte, the sporozoite begins replications, leading to the bursting of the liver cells, releasing infective merozoites (Fig 5B). The merozoites continue the life cycle by infecting red blood cells during what is termed the erythrocytic cycle of infection (Fig 5C) [25]. Within the erythrocytes, the parasites proceed to develop from trophozoites, into schizonts and finally merozoites, which are capable of reinfecting other red blood cells (Fig 5C) [25]. It is at this point that the host may experience symptoms such as sweating and chills due to the release of the parasite's waste products being released into the blood stream [24]. While some of the merozoites re-infect erythrocytes, others

develop into gametocytes (Fig 5C). When a mosquito takes a blood meal, the gametocytes in the host's blood enter the mosquito's midgut where sexual development is initiated [25]. The cycle then repeats itself through the mosquito and is transmitted to another host.

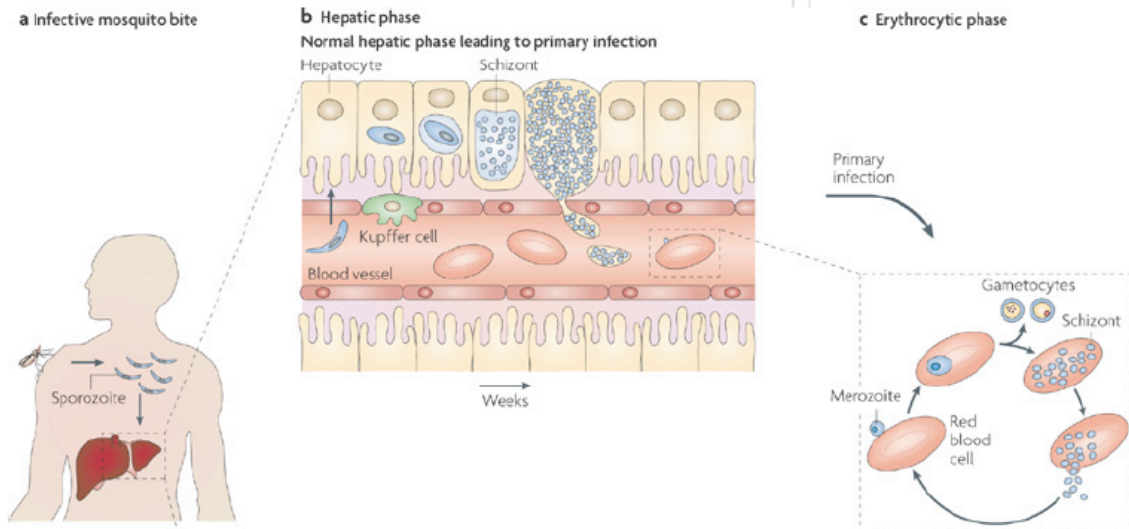


Figure 5. *Plasmodium* spp. life cycle within an infected host [23]

1.3 UTILIZATION OF COMMON HOST ENTRY FACTORS

Consequently, after studying the life cycles of both HCV and *Plasmodium*, it is clear that the liver serves as an important stage for viral and parasitic development. Furthermore, to enter host hepatocytes, both HCV and *Plasmodium* utilize common host factors such as HSPGs, CD-81, SR-B1, and ApoE.

1.3.1 Heparan sulfate proteoglycans (HSPGs)

HSPGs are linear polysaccharides occurring as proteoglycans attached closely to the hepatocytes surface [26]. Glycosaminoglycan (GAG) chains are found ubiquitously on eukaryotic cell surfaces but differ in the composition and proportion found on the cell surface depending on the host species, the cell, and tissue type [26]. These GAGs on the proteoglycans provide primary docking sites for many pathogens including HCV and *Plasmodium* [14,22].

By using recombinant envelope glycoprotein E2 and virus-like particles as ligands for cellular binding, Barth, et al. (2003) has determined that the cellular binding of HCV envelope requires E2-HSPG interaction. Throughout their study, it is shown that heparin and liver-derived highly sulfated heparan sulfate successfully inhibited the binding and entry of virus-like particles to hepatocytes; furthermore, cell surface heparan sulfate was degraded with a pretreatment of heparinases resulting in a reduction of viral envelope protein binding [14]. Taking a closer look at HCV glycoprotein virus, Barth, et al. (2003) showed that a deletion of HCV E2's hypervariable region-1 caused a reduction in E2-heparan interaction via surface plasmon resonance analysis, indicating that the N-terminus of glycoprotein E2 is comprised of positively charged residues which are important in mediating E2-HSPG binding [43].

Likewise, Coppi, et al. (2007) has also concluded that *Plasmodium*, in its sporozoite stage, migrates through cells expressing low-sulfated HSPGs, such as skin and endothelium; however cells that express high-sulfated levels of HSPGs, such as hepatocytes, activate the sporozoites for invasion of the host cell. Overall, Coppi, et al. (2007) suggested that sporozoites preferentially migrate in the presence of HSPGs with low levels of sulfation, however, when in contact with cells expressing highly sulfated HSPGs, sporozoites undergo circumsporozoite

protein (CSP) cleavage and active invasion into hepatocytes showing that HSPGs provide an environmental signal that determines *Plasmodium* sporozoite behavior (Fig 6) [22].

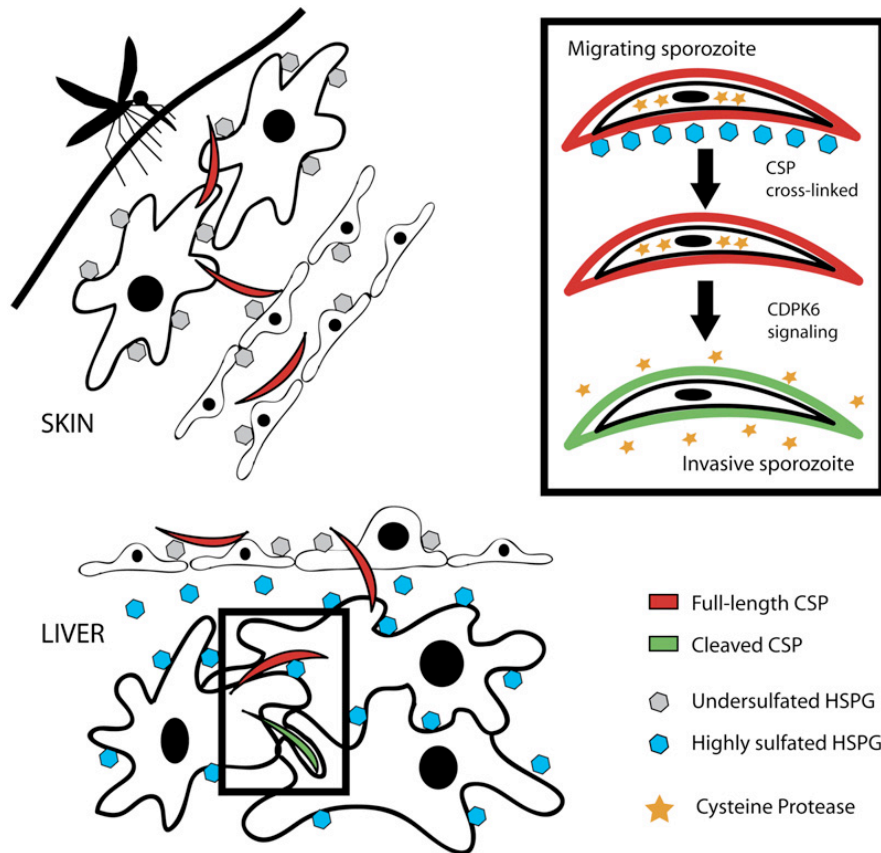


Figure 6. Model of Sporozoite Activation for Invasion by Highly Sulfated HSPGs [22]

Furthermore, by examining the CSP of *Plasmodium* sporozoites, results indicated that the NH₂-terminal portion of CSP binds to and is cross-linked by highly sulfated HSPGs causing a signaling cascade mediated by CDPK-6 [22]. Activation of CDPK-6 results in the secretion of proteases, which cleave CSP and bind the parasite to HSPGs and successful invasion [22]. All in all, both HCV and *Plasmodium* utilize high-sulfated HSPGs as an initial docking site for hepatocyte entry.

1.3.2 Cluster of Differentiation-81 (CD-81)

CD-81 is a well-known tetraspanin transmembrane protein primarily expressed on hepatocytes, T-lymphocytes, and B-lymphocytes [15,27] acting as an important mediator of signal transduction events, including cell development, activation, growth, and motility [27].

Pileri, et al. (1998) confirmed that CD-81 as the human cell surface molecule that binds HCV E2 glycoprotein by utilizing recombinant E2 and anti-CD-81 antibodies. Immunoblots and immunohistochemical stained with biotin-labeled E2 confirmed that E2 did, in fact, interact with CD-81 [15]. Furthermore, when attaching human TRX-EC2 proteins to polystyrene beads and incubating them with infectious plasma containing viral RNA molecules, then preincubating the beads with anti-CD-81, virus binding was inhibited [15]. Demonstrated by Pileri, et al. (1998), human CD-81 is capable of binding the E2 proteins on HCV as well as the viral particles suggesting it is utilized for entry into cells expressing CD-81.

Coincidentally, *Plasmodium* has also been shown to rely on CD-81, a well-known HCV receptor, to invade hepatocytes as shown by Silvie, et al (2007). While Silvie, et al. mentions that depending on the host cell type, *P. berghei* sporozoites use several distinct pathways for invasion into hepatocytes, it is still apparent that CD-81 provides one of the most efficient ways the parasite enters the host cell. Even though there are many different pathways *P. berghei* utilizes during invasion, it is clear that this may be the reason this particular species of parasite is capable of infecting a wide range of host cell types *in vitro* [23]. Furthermore, it may explain why human parasites have the ability to escape immune responses and conquer polymorphisms of host receptors [23].

1.3.3 Scavenger Receptor B1 (SR-B1)

In addition to HSPGs and CD-81, SR-B1 is an integral membrane protein found in various cell types, including hepatocytes, and has also been found to be a requirement for HCV and *Plasmodium* entry into host hepatocytes. It functions as a receptor for high-density lipoprotein and drives cholesterol from the peripheral tissues towards the liver for excretion [28]. Originally, it was thought that HCV and *Plasmodium* entry was solely dependent on CD-81; however, it was recently discovered that entry of such pathogens requires an interaction between CD-81 and SR-B1 [29].

Scarselli, et al. (2002) determined that SR-B1 interacts with HCV E2 protein in order to attach to host hepatocytes. As it was recently concluded that E2 recognition by hepatoma cells is independent from the viral isolate, however, E2-CD-81 interaction is isolate specific. Scarselli, et al. indicates that it is E2-SR-B1, identified in HepG2 cells, interaction that is very selective and responsible for the interaction between HCV E2 and the host hepatocyte. Furthermore, SR-B1 is a highly expressed receptor in liver hepatocytes, which could account for liver tropism of HCV. Scarselli, et al. (2002) even points out that when different variants are deleted in the hypervariable region 1 (HVR1), the virus is unable to recognize SR-B1, however, it can recognize CD-81. They conclude that the HVR1 is essential for the main route of HCV infection, but alternative pathways for virus entry are plausible.

Similarly, Rodrigues, et al. (2008) established that SR-B1 plays a key role in *Plasmodium* sporozoite infection as well as parasitic development within the infected hepatocytes. By utilizing RNA interference, it is shown that SR-B1 is the strongest regulator of *Plasmodium* infection in a screen of various lipoprotein-related host factors [24]. Furthermore, Rodrigues, et al.'s (2008) results indicated that inhibition of SR-B1 function reduced *P. berghei* infection in

HuH7 cells, while over-expression of SR-B1 increased parasitic infection. In addition, when SR-B1 was silenced *in vivo* and inhibited in primary hepatocytes, *Plasmodium* infection decreased significantly [24]. In conclusion, SR-B1 was found to influence sporozoite invasion into hepatocytes as well as affect parasite development within these cells [24].

1.3.4 Apolipoprotein E (ApoE)

Apolipoproteins are host carrier proteins responsible for combining lipids to form lipoprotein particles [30]. Apolipoprotein E (ApoE), more specifically, functions in the transport of triglycerides and cholesterol to the liver tissue.

Chang, et al. (2007) illustrated findings that apoE is essential for HCV infectivity and production by firstly, examining the properties of HCV virions and the role of apoE in HCV entry and assembly within host hepatocytes. Low-density HCV virions showed to be rich in apoE protein after the HCV RNA-containing particles were separated by sucrose density gradient sedimentation. By using apoE- and HCV E2-specific monoclonal antibodies, HCV virions were precipitated and HCV infectivity was neutralized efficiently by the apoE-specific antibodies [31]. Chang, et al. (2007) also used an siRNA-mediated knockdown of apoE expression where HCV production had significantly decreased. Their conclusions reveal that apoE is, in fact, required for HCV entry and manufacturing of virions in host hepatocytes [31].

Recently, Liu, et al. (2011), examined a human apolipoprotein E peptide, hEP, which contains a receptor binding fragment as well as a lipid binding fragment of apoE and its affects of HCV entry and infection [32]. Not only was this peptide shown to have no cytotoxicity, it also blocked the entry of cell culture grown HCV (HCVcc) [32]. Furthermore, it was illustrated that hEP inhibited HCV entry by directly blocking the binding of the virus to the cell surface

[32]. Overall, Liu, et al. (2011) concluded that apoE peptides block HCV entry and ultimately, inhibit HCV infection of hepatocytes.

Kelly, et al. (2007) states that *Plasmodium* sporozoites bind to HSPGs on hepatocytes are used by apoE-containing lipoproteins for uptake by the liver. Synthesized previously, apoEdp (a highly cationic peptide derivative of human apolipoprotein E, which has inhibited a range of microorganisms), was utilized to examine its activity and mechanism of action [33]. Firstly, the full-length tandem repeats peptide apoEdp showed the greatest antiviral and antibacterial action [33]. In a similar assay, apoEdp and apoEdpL-W inhibited *Plasmodium berghei* sporozoites from entering hepatocytes (Hepa 1-6 cells); furthermore, after washing the cells, apoEdpL-W peptide continued to block *P. berghei* entry into the cells, suggesting that the peptide binds to the cell surface irreversibly, thereby blocking entry [33]. Ultimately, it was concluded that apoEdp directly inactivates the sporozoites, however, apoEdpL-W most likely acts through interactions with host cell membrane [33].

2.0 RATIONALE FOR *IN VITRO* METHODS

HCV is especially permissive in HuH7.5 cell lines and its derivatives, while most *Plasmodium in vitro* infections have utilized HepG:2 cell lines in the past. In order to study a co-infection between the two pathogens, a common cell line must be confirmed. In this case, cell lines HuH7, HuH7.5, Replicon 2-3-, and Replicon 2-3+ cell lines were used to determine proper infection of both diseases in specific aim 1.

The HuH7 cell line is the base for all of the cell lines utilized throughout our experiment. Established in 1982, this frequently used cell line is a well-differentiated hepatocyte derived cellular carcinoma originally procured from a liver tumor in a 57-year-old Japanese male [34].

Derived from HuH7 hepatoma cells, HuH7.5 is highly permissive to HCV infection providing an excellent *in vitro* model to study this virus. When HCV infects hepatocytes, it activates interferon regulatory factor 3 (IRF3), producing interferon in cultured cells; however, this normal response is non-existent in cells especially permissive to HCV infection [34]. After numerous studies, it was concluded that permissiveness in these cells are due to a point mutation causing inactivation of dsRNA sensor retinoic acid-inducible gene-1 (RIG-1) [35,36]. Activation of RIG-1 by HCV RNA, triggers phosphorylations and nuclear translocation of IRF-3, activates innate anti-viral defenses [36].

Replicon cell lines 2-3- and 2-3+ were both derived from HuH7 cell lines. To achieve such a robust cell-culture system for HCV, Lohmann, et al. (1999) subgenomically inserted a

HCV replicon produced from genotype 1b HCV RNA. To allow more production of HCV RNA in the cell, Lohmann, et al. (1999) designed a selectable replicon that transduced neomycin (G418) resistance only to those cells that support HCV replication [37]. These cells are our Replicon 2-3+ cells, while those who were “cured” and were not resistant to G418, served as Replicon 2-3- cells.

In aim 2, HuH7.5 cell lines continued to serve as a model for *Plasmodium berghei* infection. In addition, HuH7.5.1 and CD-81 deficient cell lines were utilized. HuH7.5.1 are derived from the permissive HuH7.5 cell line. However, they have been found to be more permissive to HCV infection than HuH7.5 cells. Like HuH7.5 cells, HuH7.5.1 cells also have a defect in the RIG-1 pathway, but may be more permissive due to the reduced efficiency of the host cell’s innate defenses. Furthermore, HuH7.5.1 cells may have a higher density of viral receptors, which allow for easier attachment and entry into the cell [12].

The CD-81 deficient cells were kindly provided by Jana Jacobs of Dr. Tianyi Wang’s laboratory at the University of Pittsburgh. HuH7.5.1 cells were transfected with *in vitro* transcribed genomes of HCV strain JFH-1, then passaged sequentially with cells and media, as described in Russell, et al (2008) [38]. Limiting dilutions were performed on the cell and passaged 15 times.

Infections occurred for a duration of 60 hours due to the development of *Plasmodium* in the hepatocytes. Infections cannot occur after 62 hours due to the fact that the hepatocytes will burst releasing the subsequent merozoites (erythrocytic cycle) into the blood. In order to detect the active replicating virus as well as *Plasmodium* in the samples collected from each infection, primers targeting *P. berghei* 18s and the N terminus region of HCV were used. RPS11 was used

as an endogenous control for the HuH7 cell lines and its derivative cell lines. Duplicate wells were infected during each experiment and three replicate experiments were performed.

2.1 HYPOTHESIS

Given the increasing prevalence of hepatitis C in parts of the world where malaria is endemic, hepatitis C virus and *Plasmodium spp.* co-infections are a likely occurrence. In this case, we also hypothesize that co-infection with these pathogens will affect the replication of either pathogen during the liver stage. Furthermore, it is likely that *Plasmodium* parasites utilize Occludin and Claudin-1 host factors, which are important for HCV entry, to invade hepatocytes.

2.2 SPECIFIC AIMS

1. Develop a model to study *Plasmodium* and Hepatitis C virus (HCV) co-infection.
 - 1.1 Determine if co-infection in hepatocytes affects the development of either pathogen.
 - 1.1 Determine if pathogens are able to infect the same cell.
2. Examine other host factors that HCV utilizes to gain entry into host hepatocytes (e.g. Claudin-1, Occludin, and human apoE) and their affect on *Plasmodium* infection.

3.0 MATERIALS AND METHODS

3.1 SPECIFIC AIM #1: DEVELOP A MODEL TO STUDY *PLASMODIUM* AND HCV CO-INFECTION

3.1.1 Infection of hepatocytes with hepatitis C virus and *Plasmodium*

Anopheles mosquitoes infected with *P. berghei* were supplied by Dr. Photini Sinnis of New York University. Female Anopheles mosquitoes were separated from males, dissected and salivary glands, containing *P. berghei* sporozoites were collected. Sporozoites were counted and used to simultaneously infect with JFH-1 AM2 HCV, as described by Russell, et al. (2008) into hepatocyte cell lines HuH7, HuH7.5, Replicon 2-3-, and Replicon 2-3+, which were seeded in 48-well plates at a density of 75,000 cells per well; these experiments were done in collaboration with Dr. Tianyi Wang [38]. Infections also included a negative control (hepatocytes only), a *P. berghei* infection only and a HCV infection only in each cell line. For infection 40,000 *P. berghei* sporozoites were infected simultaneously with an approximate ratio of HCV to cells seeded equal to 1. Chamber slides were also prepared with each cell line the day prior to infection at a cell density of 50,000 cells per well, infected with 40,000 *P. berghei* sporozoites and an HCV:cells seeded ratio of 1:1. Following infection, hepatocytes were centrifuged for 5

minutes at 2,000RPM. Media, Dulbecco's Modified Eagle Media (DMEM) with 10% Fetal Bovine Serum (FBS), 10mL of Penicillin/Streptomycin, 5mL L-Glutamine, and 5mL Amphotericin B for 500mL of complete DMEM media, was changed after 4 hours and every 24 hours until harvested with TRIzol (Invitrogen) at 60 hours. Chamber slides were fixed with 4% paraformaldehyde at 60 hours.

3.1.2 Immunofluorescent staining and analysis of chamber slides

Chamber slides were prepared and infected during all experiments as previously described. To stain the samples with antibodies, samples in the chamber slides were first incubated for 15 minutes with a permeability solution consisting of 0.1% Triton X-100 in Phosphate Buffered Saline (PBS), then washed three times with PBS. The samples were then blocked with a blocking buffer comprised of 2% Bovine Serum Albumin (BSA) in PBS for one hour. Following the incubation for an hour, samples were washed five times with 2% blocking buffer before primary antibodies were added. Primary antibodies consisting of a 1:200 dilution of anti-HCV E2 (rat), 1:40 dilution of anti-*Plasmodium* HSP70 (mouse) and 2% blocking buffer solution were placed on the samples and incubated for 2 hours at room temperature or overnight at 4°C. Primary antibodies were removed and saved; the samples were washed five times with 2% blocking buffer solution. Following primary antibody incubation, secondary antibodies were made with 1:400 dilution of Alexa Fluor-488 goat anti-rat IgG, 1:400 dilution of Alexa Fluor-594 goat anti-mouse IgG and 2% blocking buffer solution. Secondary antibodies were incubated for 45 minutes to one hour at room temperature, and then discarded. A 1:2000 DAPI (4', 6-diamidino-2-phenylindole) solution was added to each sample to incubate for 5 minutes at room

temperature. Lastly, Prolong Gold Anti-fade was added to the slide, covered with a cover slip and sealed with clear nailpolish.

3.1.2.1 Immunofluorescent pictures

Pictures were taken at the University of Pittsburgh's Center for Biological Imaging with the Olympus Provis III microscope, utilizing Magnafire version 2.B1 software. The amount of parasites were counted and the area of each parasite was taken for comparison among the cell lines.

3.1.3 RNA Isolation from all infected samples

To isolate the RNA from the samples obtained from the infection, the Invitrogen TRIzol protocol was followed. Ambion's TURBO DNase treatment was performed on each sample to further clean the samples; the TURBO DNase protocol was followed. Furthermore, a PCR reaction utilizing New England BioLabs Quick-Load Taq 2X Master Mix was performed to confirm the absence of contaminating genomic DNA. The RNA concentration was measured and all samples were diluted to 300 ng/ μ L. After diluting, the cDNA was made of all RNA samples via Invitrogen's SuperScript III First-Strand Synthesis SuperMix cDNA synthesis kit and protocol.

3.1.4 Preparation of relative standards for real-time PCR

Standards for Real-time PCR (qRT-PCR) consisted of an initial ratio of 1:1:10 of *Plasmodium* MRA871:HCV JFH-1 AM2:HuH7.5 cDNA, referred to as "stock". cDNA was previously

synthesized from isolated mRNA using Invitrogen's Superscript III 1st strand synthesis SuperMix and protocol.

Table 1. Specific target (primer) sequences for aim 1.

Target		Sequence
Pb18s	Forward	5' - AAG CAT TAA ATA AAG CGA ATA CAT CCT TAC - 3'
	Reverse	5' - GGA GAT TGG TTT TGA CGT TTA TGT G - 3'
HCV	Forward	5' - AGCGTCTAGCCATGGCGTT -3'
	Reverse	5' - GCAAGCACCCCTATCAGGCAGT - 3'
RPS11	Forward	5'- GTGACCTTGAGCACGTTGAA - 3'
	Reverse	5' - CAAGTACAACCGCTTCGAGA - 3'

After optimizing for each individual target (primer) (Table 1), it was confirmed that optimal standards for PB18s primers were diluted from the 1:1:10 stock standard to 1:10, 1:100, 1:1000, and 1:5000, optimal standards for HCV primers were diluted from the 1:1:10 stock standard to 1:200, 1:40,000, 1:160,000, and 1:320,000, and optimal standards for RPS11 primers, used as an endogenous control, were diluted from the 1:1:10 stock standard to 1:1, 1:50, 1:100 and 1:500. In order for Applied Biosystem's Step-One Plus qRT-PCR instrument and software to quantify the amount of replication products were in each sample, an arbitrary unit was assigned to each of the dilutions used in the standard curve as seen in Table 2. To determine the arbitrary unit, a 1:1 dilution (stock 1:1:10 MRA871:HCV JFH-1 AM2:HuH7.5 cDNA) was equal to 100, 1:10 dilution of the stock was set equal to 10 (100/10), 1:100 dilution of the stock was set to 1

(100/100), etc. Standard dilutions were aliquoted to ensure no occurrence of freeze-thaw degradation.

Table 2. Arbitrary Unit Assignments for qRT-PCR standard curve (Aim 1).

Primer Target	cDNA Relative Dilution (From 1:1:10 Stock)	Arbitrary Unit Assigned
PB18S rRNA	1:10	10
	1:100	1
	1:1000	0.1
	1:5000	0.02
HCV 5' UTR	1:200	0.5
	1:40,000	0.0025
	1:160,000	0.000625
	1:320,000	0.000313
RPS11	1:1	100
	1:50	2
	1:100	1
	1:1000	0.1

3.1.5 Quantitative Real-Time Polymerase Chain Reaction and Analysis

Prepared in a Fast-Optical PCR 96-well plate from Applied Biosystems, 4 μ L of sample, standards and no-template controls were placed in separate wells. Three “cocktails” consisting of 5 μ L Power SybrGreen Mix (Applied Biosystems), 0.1 μ L forward primer, 0.1 μ L reverse primer, and 2.8 μ L of nucleus-free water were made for each well for the three targets: HCV 5'UTR, *P. berghei* 18S rRNA and RPS11 (endogenous control). A total of 10 μ L of SybrGreen cocktail including the template was in each well. The reaction was processed in a StepOne Plus Real-Time PCR System (Applied Biosystem) where StepOne Software v2.1 was used to record data. The standard method used to amplify the products was first to heat to 95°C for 10 minutes, cycle 95°C for 15 seconds and 60°C for 1 minute 40 times, heat once again to 95°C for 15

seconds, cool to 60°C for 1 minute, and heat to 95°C for 15 seconds to produce a melt curve. Quantities of both HCV 5'UTR and *P. berghei* 18S rRNA were normalized to quantities of RPS11 (Equation 1). The quantities of all three amplified products (RPS11, HCV 5' UTR, and PB18S rRNA) were obtained by interpolation from the standard curves from using individual primer pairs on the standard cDNA for PCR.

Equation 1. Normalization equation based on RPS11 qRT-PCR quantities.

A. $(\text{Quantity of HCV Sample}) / (\text{Quantity of RPS11 Sample})$

B. $(\text{Quantity of PB18S Sample}) / (\text{Quantity of RPS11 Sample})$

3.1.6 Dose Response Infection

Of the four cell lines utilized in the first stage of this study, HuH7.5 proved to have the highest *P. berghei* and HCV co-infection and so was used throughout the remainder of this study. HuH7.5 cells were seeded at 75,000 cells per well in 48-well plates one day prior to infection. To test different doses of each pathogen and the overall affect of replication of each pathogen, HuH7.5 cell only served as a negative control in this experiment, while an infection of 40,000 sporozoites to 75,000 cells and an HCV:cells seeded ratio of 1:1 served as a positive control for a co-infection. Amounts of parasite and virus were varied one at a time, while the other pathogen infection amount was kept static, as shown in Table 3.

Table 3. Dose Response Infection with varying amounts of HCV and *P. berghei*.

	Amount of <i>P. berghei</i> sporozoites added	Approximate ratio of HCV added:HuH7.5 cells seeded
HuH7.5 Cells Only	N/A	N/A
HuH7.5 <i>P. berghei</i> + HCV	40,000 sporozoites/well	1:1
HuH7.5 ½ HCV + <i>P. berghei</i>	40,000 sporozoites/well	0.5:1
HuH7.5 ¼ HCV + <i>P. berghei</i>	40,000 sporozoites/well	0.25:1
HuH7.5 ½ <i>P. berghei</i> + HCV	20,000 sporozoites/well	1:1
HuH7.5 ¼ <i>P. berghei</i> + HCV	10,000 sporozoites/well	1:1

Following infection, hepatocytes were centrifuged for 5 minutes at 2,000RPM. Media, DMEM with 10% FBS, 10mL of Penicillin/Streptomycin, 5mL L-Glutamine, and 5mL Amphotericin B for 500mL of complete DMEM media, and was changed after 4 hours and every 24 hours until harvested with TRIzol (Invitrogen) at 60 hours. For the duration of the infection, cells and samples were incubated at 37°C with 5% CO₂. RNA was extracted from samples, as previously noted, and qRT-PCR was performed to analyze the samples.

3.1.7 Statistical Analysis

Figures were configured to show a percentage of the control (*P. berghei* infection only or HCV infection only) and a comparison was made to the samples with an established co-infection. The control was set as 100%. Statistical analysis was performed on biological duplicates and technical duplicates for each sample using Prism 5. The standard deviation of the mean was calculated using this program and included in each figure. A student t-test (unpaired, two-sided) was performed to determine a significant difference between samples, where significance was noted as $p < 0.05$.

3.2 SPECIFIC AIM #2: EXAMINE OTHER HOST FACTORS HCV UTILIZES TO GAIN ENTRY INTO HOST HEPATOCYTES (E.G. CLAUDIN-1, OCCLUDIN AND HUMAN APOE) AND THEIR AFFECT ON *PLASMODIUM* INFECTION

3.2.1 Transfection of Claudin-1 and Occludin siRNA into hepatocytes

HuH7.5 cells were seeded at a density of 5,000 cells per well in a 48-well plate two days prior to transfection in DMEM media with 10% FBS, 10mL Penicillin/Streptomycin, 5mL L-glutamine, and 5mL Amphotericin B. Invitrogen's siRNA Lipofectamine protocol was followed for a 48-well plate. Dilutions to 6pM were made of each siRNA, including a positive "scramble" siRNA control and added approximately 24 hours prior to infection, as described by Liu, et al. (2009) [39]. Cells were 30-50% confluent at the time of transfection. The *P. berghei* infection consisted of negative controls (HuH7.5 cells only) and an infection of 40,000 *P. berghei* sporozoites were performed for each siRNA. Cells and samples were incubated throughout the entirety of transfection and infection at 37°C with 5% CO₂.

3.2.2 Utilizing CD-81 deficient cells to study *P. berghei* infection

CD-81 has been shown to be a required host factor to ensure *Plasmodium* infection, however, *P. berghei*'s entry is not dependent on this particular host factor. Recently, Jana Jacobs of Dr. Tianyi Wang's lab, obtained an HuH7.5.1 derivative line that is deficient in CD-81 by extended passages similarly described by Russell, et al (2008) [38]. CD-81 deficiency was confirmed via flow cytometry performed by Jacobs. HuH7.5.1 and CD-81 deficient cells (CD-81-) were seeded at 5,000 cells per well in a 48-well plate simultaneously with HuH7.5 cells used for the

siRNA transfection experiment. At the time of infection, they were approximately 75-80% confluent. After infection with 40,000 *P. berghei* sporozoites, samples were centrifuged at 2,000RPM for 5 minutes and placed in a 37°C incubator with 5% CO₂. Media was changed after 3-4 hours following infection and every 24 hours thereafter. HuH7.5.1 cells only, CD-81- cells only and CD-81- cells including *Plasmodium* sporozoites samples were obtained after 60 hours. Cells and samples were incubated during infection at 37°C with 5% CO₂.

3.2.3 Addition of human ApoE peptide into hepatocytes

A dilution of 10pM of Human ApoE peptide and positive control “scramble” peptide, obtained from Dr. Tianyi Wang’s lab, was added at the time of infection. Peptide addition was performed in 48-well plate as well as chamber slides for Immunofluorescent analysis. hEP was added simultaneously with 40,000 *P. berghei* sporozoites. Following infection with *P. berghei* sporozoites and hEP, samples were centrifuged at 2,000RPM for 5 minutes. Complete DMEM media (with all antibiotics previously mentioned) was changed 3-4 hours following infection and every 24 hours thereafter until harvested or fixed at 60 hours. Cells and samples were incubated during infection at 37°C with 5% CO₂.

3.2.4 RNA Isolation from all infected samples

To isolate the RNA from the samples obtained from the infection, the Invitrogen TRIzol protocol was followed. Ambion’s TURBO DNase treatment was performed on each sample to further clean the samples; the TURBO DNase protocol was followed. Furthermore, a PCR reaction utilizing New England BioLabs Quick-Load Taq 2X Master Mix was performed to confirm there

was no genomic DNA contamination. The RNA concentration was measured and all samples were diluted to 300 ng/μL. After diluting, the cDNA was made of all RNA samples via Invitrogen’s SuperScript III First-Strand Synthesis SuperMix cDNA synthesis kit and protocol.

3.2.5 Preparation of relative standards for real-time PCR

Standards for RT-PCR consisted of an initial ratio of 1:1:10 of *Plasmodium* MRA871:HCV JFH-1 AM2:HuH7.5 cDNA, referred to as “stock”. cDNA was previously synthesized from isolated mRNA using Invitrogen’s Superscript III First-strand synthesis SuperMix and protocol. After optimizing for each individual target (primer), it was confirmed that optimal standards for PB18S primers were diluted from the 1:1:10 stock standard to 1:10, 1:100, 1:1000, and 1:5000 and optimal standards for RPS11 primers, used as an endogenous control, were diluted from the 1:1:10 stock standard to 1:1, 1:50, 1:100 and 1:500. Claudin-1 and Occludin primers were made from sequences mentioned in Han, et al. (2004) [40].

Table 4. Specific target (primer) sequences for aim 2.

Target		Sequence
Pb18s	Forward	5' - AAG CAT TAA ATA AAG CGA ATA CAT CCT TAC – 3'
	Reverse	5' - GGA GAT TGG TTT TGA CGT TTA TGT G – 3'
Claudin-1	Forward	5' – AGC CAG GAG CCT CGC CCC GCA GCT G – 3'
	Reverse	5'- CGG GTT GCC TGC AAA GT -3'
Occludin	Forward	5' – GGC GCA TAT ACA GAC CCA AGA G -3'
	Reverse	5' – GAT AAT CAT GAA CCC CAG GAC AAT – 3'
RPS11	Forward	5' - GTG ACC TTG AGC ACG TTG AA – 3'
	Reverse	5' – CAA GTA CAA CCG CTT CGA GA – 3'

In order for Applied Biosystem's Step-One Plus qRT-PCR machine and software to quantify the amount of replication products were in each sample, an arbitrary unit was assigned to each of the dilutions used in the standard curve as seen in Table 4. To determine the arbitrary unit, a 1:1 dilution (stock 1:1:10 MRA871:HCV JFH-1 AM2:HuH7.5 cDNA) was equal to 100, 1:10 dilution of the stock was set equal to 10 (100/10), 1:100 dilution of the stock was set to 1 (100/100), etc. Standard dilutions were aliquotted to ensure no occurrence of freeze-thaw degradation.

Table 5. Arbitrary Unit Assignments for qRT-PCR standard curve (Aim 2).

Primer Target	cDNA Relative Dilution (From 1:1:10 Stock)	Arbitrary Unit Assigned
PB18S rRNA	1:10	10
	1:100	1
	1:1000	0.1
	1:5000	0.02
RPS11	1:1	100
	1:50	2
	1:100	1
	1:1000	0.1

3.2.6 Quantitative Real-Time Polymerase Chain Reaction and Analysis.

Prepared in a Fast-Optical PCR 96-well plate from Applied Biosystems, 4 μ L of sample, standards and no-template controls were placed in separate wells. Two "cocktails" consisting of 10 μ L SybrGreen Premix ExTaq II 2X (Applied Biosystems), 0.1 μ L forward primer, 0.1 μ L reverse primer, 0.4 μ L ROX Reference Dye (Applied Biosystems), and 2.7 μ L of nucleus-free water were made for each well for the two targets: *P. berghei* 18S rRNA and RPS11 (endogenous control). A total of 20 μ L of SybrGreen cocktail including the template was in each

well. The reaction was processed in a StepOne Plus Real-Time PCR System (Applied Biosystem) where StepOne Software v2.1 was used to record data. The standard method used to amplify the products was first to heat to 95°C for 10 minutes, cycle 95°C for 15 seconds and 60°C for 1 minute 40 times, heat once again to 95°C for 15 seconds, cool to 60°C for 1 minute, and heat to 95°C for 15 seconds to produce a melt curve. Quantities of *P. berghei* 18S rRNA were normalized to quantities of RPS11 and compared. Primers for Claudin-1 and Occludin were used to ensure knock down of these host factors; they were normalized to levels of RPS11 during analysis of qRT-PCR.

Equation 2. Normalization equation based on RPS11 qRT-PCR quantities.

$$\frac{\text{(Quantity of PB18S Sample)}}{\text{(Quantity of RPS11 Sample)}}$$

3.2.7 Specific Taqman Primer Probe qRT-PCR

Taqman Zen primer probe (IDTDNA) in addition to PB18S and RPS11 primers were used in Claudin-1, Occludin, and hapoE samples to ensure a more specific quantification of amplified products and minimize the background amplification observed when using the SybrGreen qRT-PCR assay (Table 6).

Table 6. IDT primer probe sequences and locations.

Target		Sequence
Pb18s	Forward	5' - AAG CAT TAA ATA AAG CGA ATA CAT CCT TAC - 3'
	Reverse	5' - GGA GAT TGG TTT TGA CGT TTA TGT G - 3'
RPS11	Forward	Assay ID: Hs.PT.47.3418445.g Gene Symbol:
	Reverse	RPS11 Species: Human Reference Sequence: NM_001015(1) (Ho mo sapiens ribosomal protein S11 (RPS11), mRNA) Exon Location: 2-3

Roche Universal Fast-Start Master Mix was prepared in a cocktail of 0.25 μ L primer probe, 5 μ L Master Mix, 2.75 μ L nuclease-free water, and 2 μ L sample cDNA per well. In order to quantify the amount of product in each sample, a standard curve was established using a 1:10 ratio of MRA871:HuH7.5 cDNA as a stock, and further diluting 1:1, 1:50, 1:500, and 1:1000 for the standard curve. The standard method used to amplify the products was first to heat to 95°C for 10 minutes, cycle 95°C for 15 seconds and 60°C for 1 minute for 50 cycles, heat once again to 95°C for 15 seconds, and finally, cool to 60°C for 1 minute.

In order for Applied Biosystem's Step-One Plus qRT-PCR instrument and software to quantify the amount of replication products were in each sample, an arbitrary unit was assigned to each of the dilutions used in the standard curve as seen in Table 7.

Table 7. Arbitrary Unit Assignments for Specific Primer probes with Zen quencher for qRT-PCR standard curve (Aim 2).

Primer Target	cDNA Relative Dilution (From 1:10 Stock)	Arbitrary Unit Assigned
PB18S rRNA	1:1	100
	1:50	2
	1:500	1
	1:1000	0.1
RPS11	1:1	100
	1:50	2
	1:100	1
	1:1000	0.1

To determine the arbitrary unit, a 1:1 dilution (stock 1:1:10 MRA871:HCV JFH-1 AM2:HuH7.5 cDNA) was equal to 100, 1:10 dilution of the stock was set equal to 10 (100/10), 1:100 dilution of the stock was set to 1 (100/100), etc. Quantities of *P. berghei* 18S rRNA were normalized to quantities of RPS11 and compared (Equation 3).

Equation 3. Normalization equation based on RPS11 qRT-PCR quantities.

$\frac{\text{(Quantity of PB18S Sample)}}{\text{(Quantity of RPS11 Sample)}}$

3.2.8 Statistical Analysis

Figures were configured to show a percentage of the control (*P. berghei* infection only) and a comparison was made to the samples with an established co-infection. The control was set as 100%. Statistical analysis was performed on biological duplicates and technical duplicates for each sample using Prism 5. The standard deviation of the mean was calculated using this

program and included in each figure. A student t-test (unpaired, two-sided) was performed to determine a significant difference between samples, where significance was noted as $p < 0.05$.

4.0 RESULTS

4.1 AIM 1: DEVELOP A MODEL TO STUDY *PLASMODIUM* AND HCV CO-INFECTION

4.1.1 Determine if co-infection in hepatocytes affects the development of either pathogen

In order to evaluate how each pathogen affects one another during a co-infection in host hepatocytes, a proper *in vitro* model had to be established. In the past, *Plasmodium spp.* have been heavily studied in HuH7 hepatoma cells, while hepatitis C virus has been primarily studied in HuH7.5 hepatoma cell lines and its derivatives due to the cell lines permissivity. Because HCV does not infect HepG:2 hepatocytes and in order to establish a model in which both *Plasmodium berghei* and hepatitis C virus would replicate, this *in vitro* study was performed in HuH7 hepatoma cells and its derivatives: HuH7.5, Replicon 2-3- and Replicon 2-3+ cells.

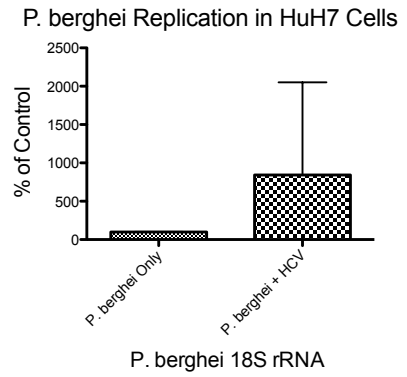
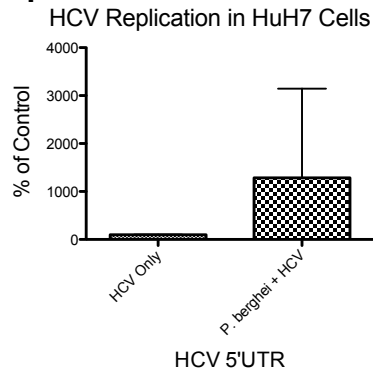
As shown (Figs 7-9), both *P. berghei* and HCV were able to replicate in each of the four cell lines selected. Three independent experiments were performed with biological duplicates and technical duplicates during analysis via qRT-PCR (Fig 7-9). Quantities of *P. berghei* rRNA and HCV 5'UTR amplified products were based on a normalized relative curve.

Each independent experiment is shown in order to illustrate the variable results during a co-infection with *P. berghei* and HCV. Two-sided, unpaired t-tests assuming a 95% confidence

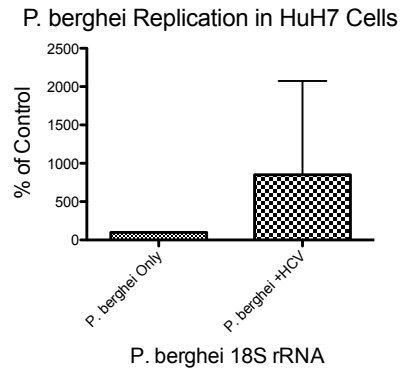
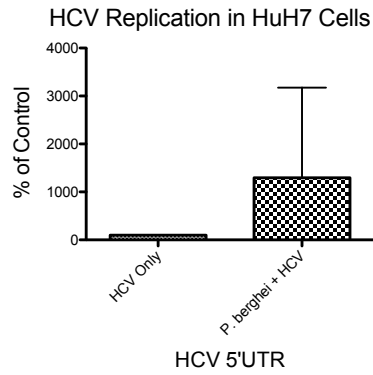
interval was performed between HCV versus *P. berghei* + HCV and *P. berghei* versus *P. berghei* + HCV in all cell lines, as well as HCV versus *P. berghei* + additional HCV in Replicon 2-3+ cell lines. When evaluating each independent experiment separately, HCV significantly decreased (p-value= <0.0001 , 0.0218) when co-infected with *P. berghei* in HuH7.5 hepatoma cells (Fig 8), and *P. berghei* significantly increased (P-values = 0.0067, 0.0224, 0.0109) in Replicon 2-3+ hepatoma cells with additional HCV in a cell line where HCV had already established an infection within the cell line (Fig 9).

After observing three independent experiments, the results seemed to be extremely varied, there were similar consistencies and trends in each individual cell line and trends among experiments for each cell line. Figure 8 shows HCV consistently decreases when *P. berghei* is present in the same sample, while *P. berghei* consistently increases when co-infected with HCV in HuH7 cells. Similar trends of HCV decreasing when co-infected with *P. berghei* were noted in Experiments 1 and 3 of Figure 8. Figure 9 also shows consistency of experiments taking place in HuH7.5 cells. Similarly, HCV decreased when in the presence of *P. berghei*, while *P. berghei* replication increased when co-infected with HCV. In replicon cells (Fig 10), results show experiments 1 and 2 to be very consistent and generally, *P. berghei* decreases when HCV has already established itself within the cell. Even though there is variability in the samples, this variability could be due to the variability of the sporozoite infectivity.

A. Experiment #1



B. Experiment #2



C. Experiment #3

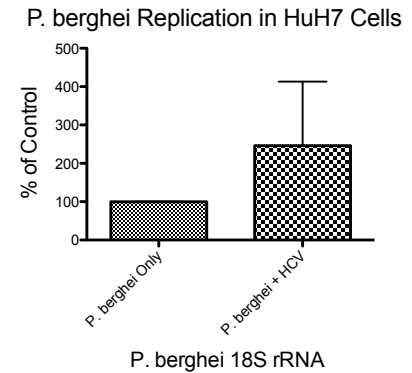
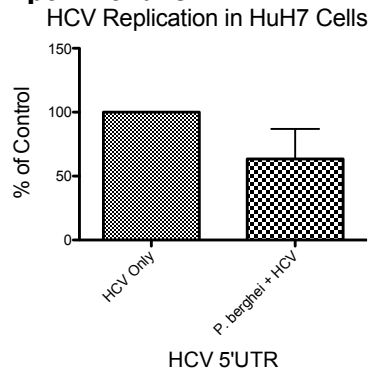
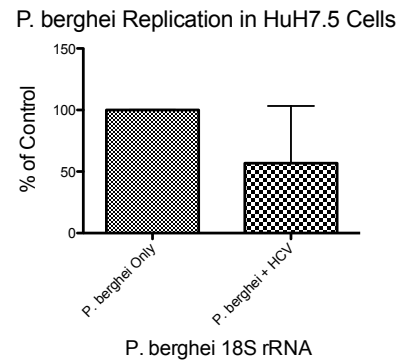
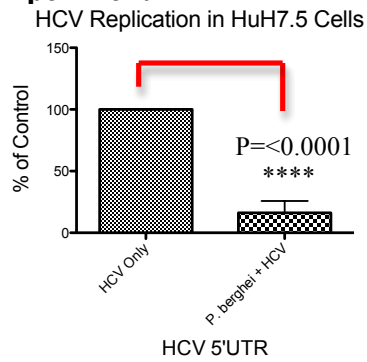
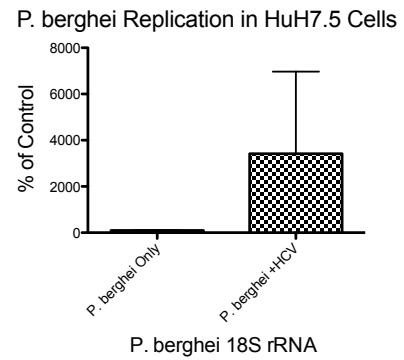
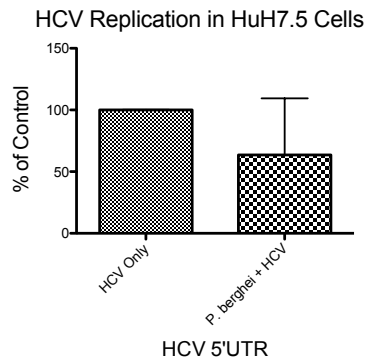


Figure 7. Quantitative RT-PCR results of three independent experiments HuH7 cells

A. Experiment #1



B. Experiment #2



C. Experiment #3

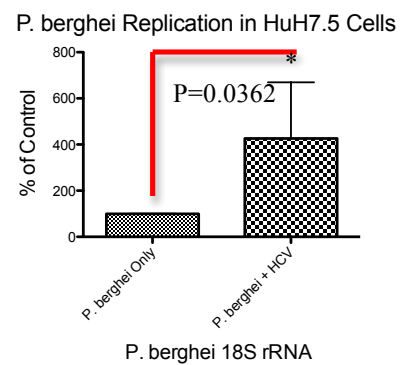
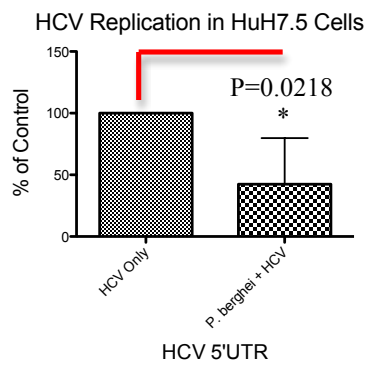
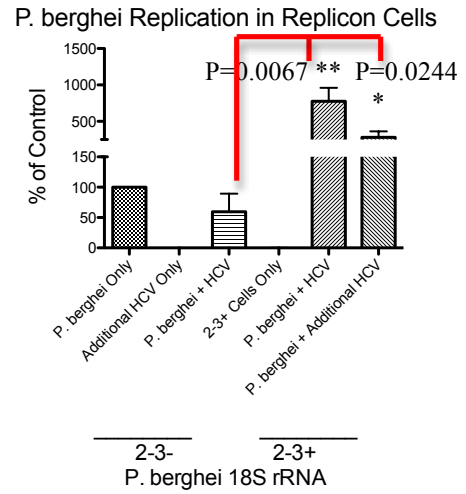
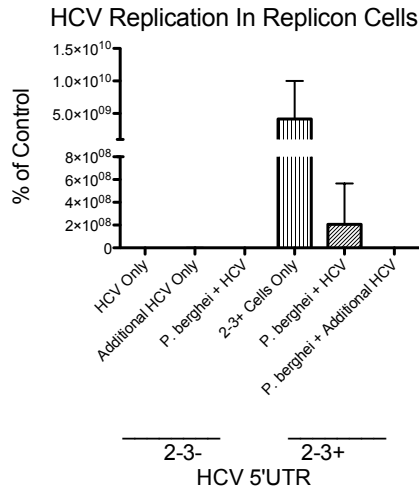
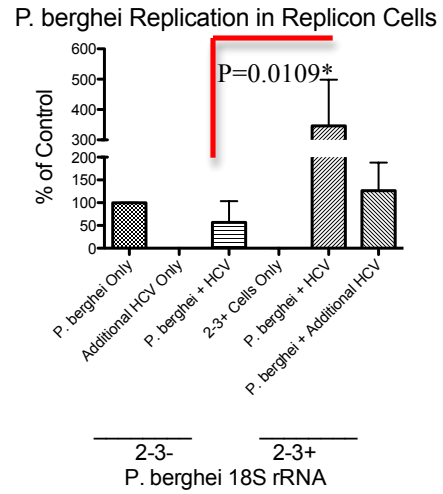
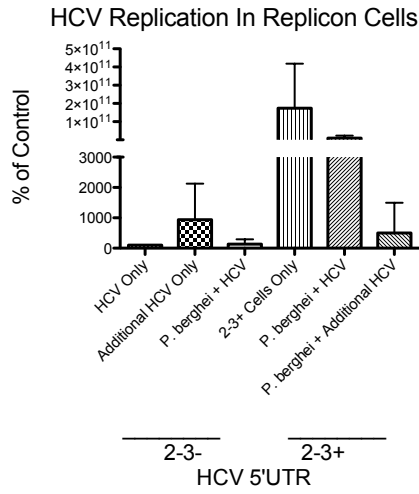


Figure 8. Quantitative RT-PCR results of three independent experiments in HuH7.5 cells

A. Experiment #1



B. Experiment #2



C. Experiment #3

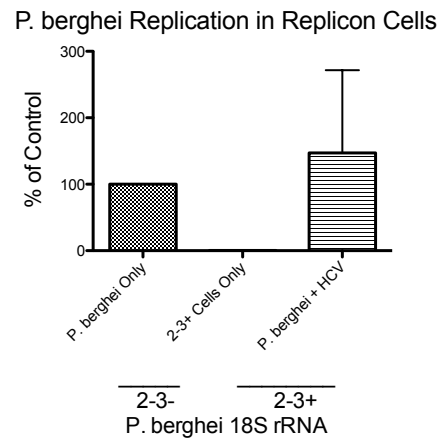
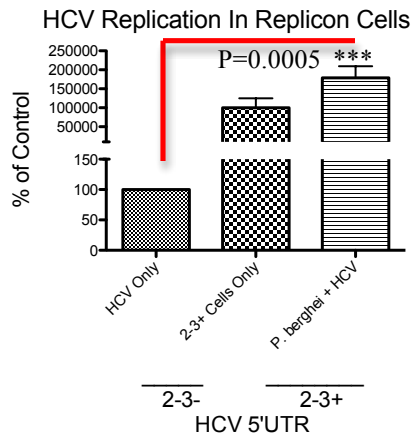


Figure 9. Quantitative RT-PCR results of three independent experiments in Replicon cells

Upon completion of the co-infection experiment (Fig 7-9), determining a cell line to utilize for further experimentation was needed. After performing an immunofluorescence assay on each cell line infected with *P. berghei*, sporozoites (liver stage parasites) were counted and measured to determine which cell line was the most permissible and susceptible to *P. berghei* infection as well as producing parasites with similar sizes. The measurement of the area for each parasite in each cell line were noted and compared (Fig 10).

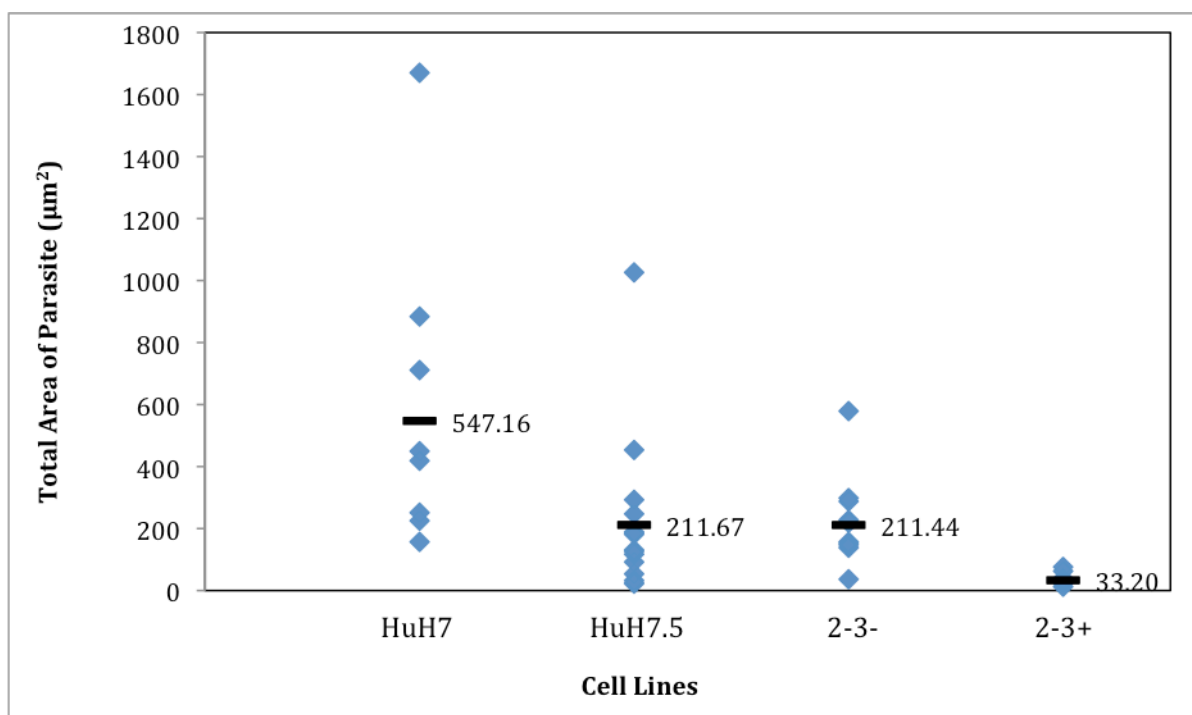


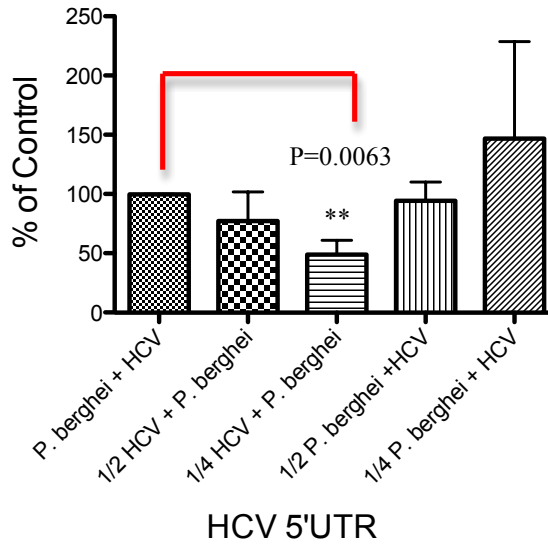
Figure 10. Comparison of *P. berghei* parasite area in each cell line

As shown in Figure 10, each point represents the area of one parasite found in each of the four cell lines (HuH7, HuH7.5, Replicon 2-3-, and Replicon 2-3+). The black dash represents the average total area in µm² of parasites found in that particular cell line when infected with *Plasmodium berghei* only. Parasites developing in HuH7 cells had an average total area of 547.16µm² and very few parasites were ultimately able to develop within these cells

(approximately 20 parasites/well). On the other hand, many parasites were able to infect and develop in HuH7.5 hepatocytes (approximately 85 parasites/well), but produced much smaller parasites when compared to HuH7-developed parasites. Both Replicon 2-3- and 2-3+ hepatocytes provided a stable environment for *P. berghei* to develop, producing parasites with an average total area of $211.44\mu\text{m}^2$ for parasites developed in Replicon 2-3- hepatocytes, and $33.20\mu\text{m}^2$ for parasites developed in Replicon 2-3+ hepatocytes. Essentially, HuH7.5 and Replicon 2-3- cell lines are the same, both “cured” of HCV infection; Figure 10 supports this idea because *P. berghei* parasites develop to the same average size and allow for similar production of the parasites. Of Replicon 2-3+ hepatocytes observed in one experiment, roughly 1% encompassed a co-localization of HCV and *P. berghei*. HuH7.5 was utilized for further experiments due to the permissive nature of the cell line to support both HCV and *P. berghei* infection.

Three independent dose response experiments were performed in HuH7.5 hepatoma cells, one independent experiment is shown (Fig 11). While HCV significantly decreased (P-value = 0.0063), as it should, when less HCV is added during infection, there was no significant difference when *P. berghei* concentrations were varied. Similarly, *P. berghei* replication significantly decreased when less sporozoites were added during infection (P-values = 0.0080, 0.0035); however, there was no significant difference in parasite replication when varying the concentration of HCV during infection. It is clear that HCV replicated better without *P. berghei* co-infection (Fig 11A); however, despite the fact that there is an increase in *Plasmodium* infection, the dose-response indicates that this increase is not sustained with decreased numbers of HCV co-infected with *P. berghei* (Fig 11B).

A. HCV Replication with Varied Doses of HCV and *P. berghei* Infection



B. *P. berghei* Replication with Varied Doses of HCV and *P. berghei* Infection

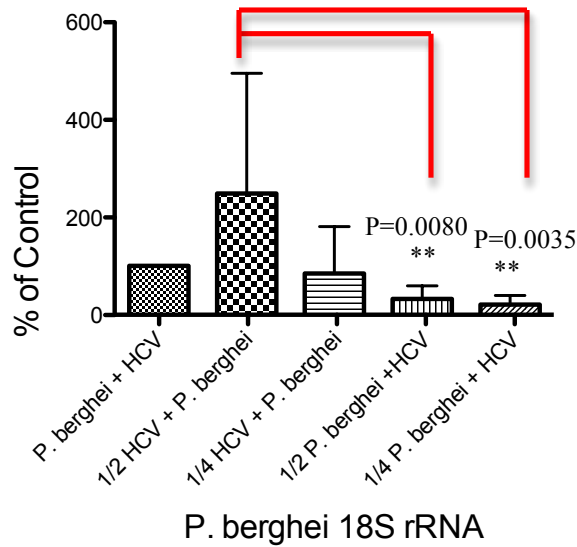


Figure 11. Quantitative RT-PCR results showing *P. berghei* and HCV replication in HuH7.5 cells with varying concentrations of *P. berghei* and HCV

Overall, it can be seen that *P. berghei* hinders the development of HCV during co-infection, but it is unclear whether HCV impacts *P. berghei* development.

4.1.2 Determine if pathogens are able to infect the same cell

It is a fact that *Plasmodium spp.* and HCV both infect liver cells; therefore, it is plausible that they could infect and replicate within the same cell. Immunofluorescent analysis targeting the E2 glycoprotein on HCV (green, Fig 12, Column 2) and the HSP70 in *P. berghei* (red, Fig 12, Column 3) provide a visual into co-localization (Merge, Fig 12, Column 4) of these two pathogens in the same cell when observing infection in HuH7 (Fig 12, Row 1), HuH7.5 (Fig 12, Row 2), Replicon 2-3- (Fig 12, Row 3), and Replicon 2-3+ (Fig 12, Row 4) cell lines. Ultimately, co-localization was seen in each cell line with the exception of HuH7 (Fig 12, Row 1, Column 2).

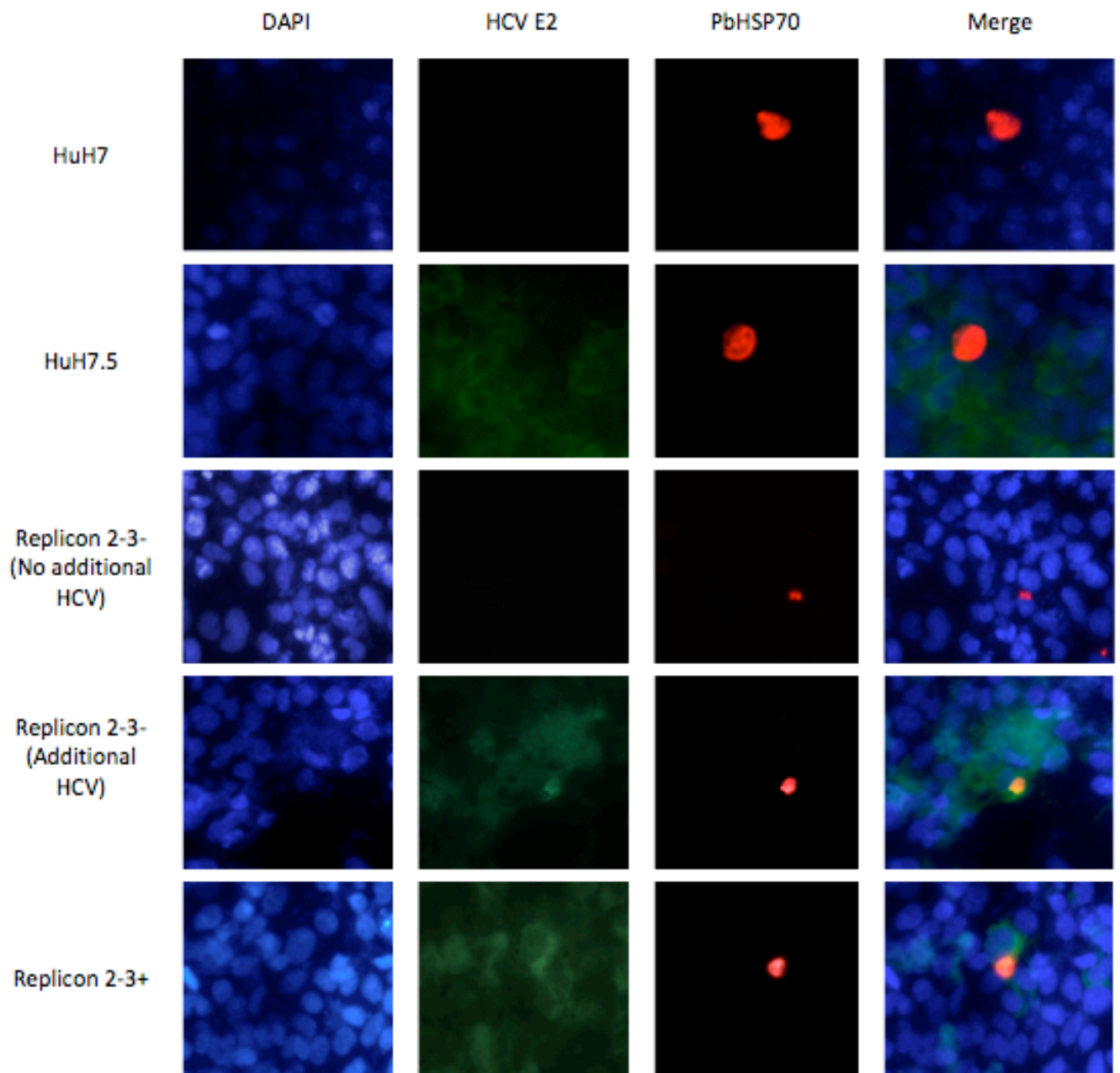


Figure 12. Immunofluorescent Analysis of co-infections in HuH7, HuH7.5, Replicon 2-3- with no additional HCV, Replicon 2-3- with additional HCV, and Replicon 2-3+ cell lines

4.2 AIM #2: EXAMINE OTHER HOST FACTORS HCV UTILIZES TO GAIN ENTRY INTO HOST HEPATOCYTES (E.G. CLAUDIN-1, OCCLUDIN AND HUMAN APOE) AND THEIR AFFECT ON *PLASMODIUM* INFECTION

4.2.1 Examining *Plasmodium berghei* replication during Claudin-1 and Occludin siRNA knockdowns

In order to observe whether entry of *P. berghei* sporozoites was inhibited by knocking down Claudin-1 and Occludin, qRT-PCR was performed on three independent experiments. HuH7.5 cells only were used as a negative control, as well as HuH7.5 cells with only Claudin-1 and Occludin siRNA added. A scramble siRNA targeting a random sequence during transfection was used as a positive control. An unpaired, two-sided t-test was performed to compare the amount of *P. berghei* 18S rRNA when Claudin-1 and Occludin siRNA was transfected compared to when no siRNA was used in the experiment (*P. berghei* infection only).

Following statistical analysis of the three independent experiments, it can be concluded that *P. berghei* replication decreases when Claudin-1 and Occludin are knocked down separately as shown in Figures 13-15 (P-value_{CLAUDIN-1} = <0.0001; P-value_{OCCLUDIN} = <0.0001). Error bars represent sample standard deviation.

P. berghei Replication with CLN-1 and OCC knockdowns

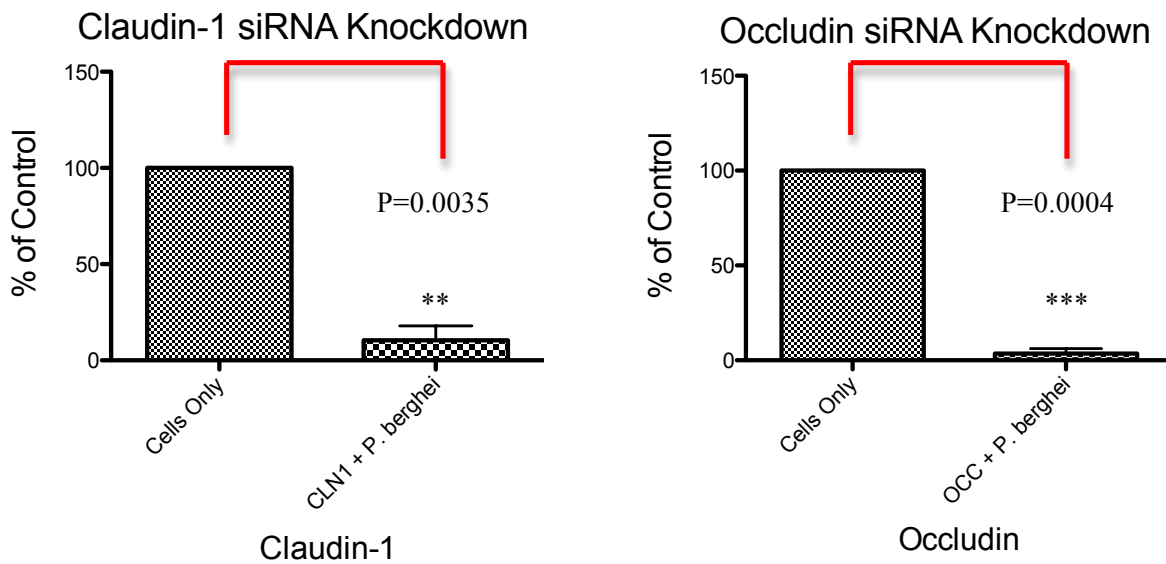
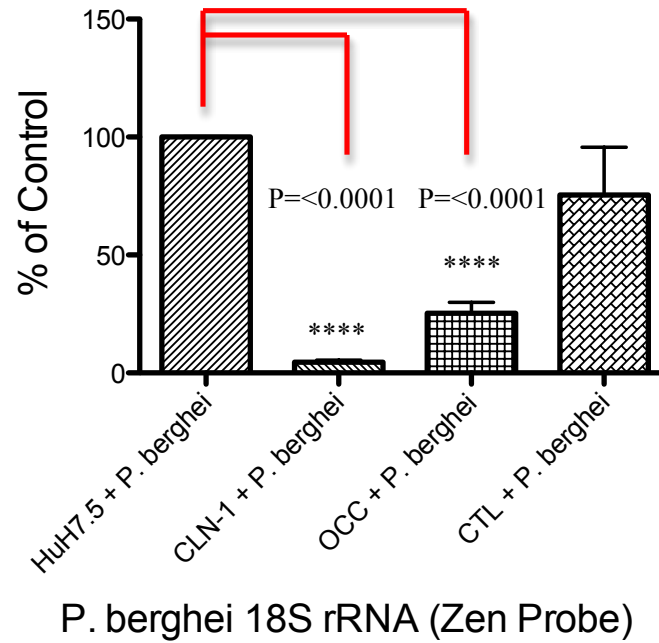


Figure 13. Experiment 1: Quantitative RT-PCR results for *P. berghei* replication in HuH7.5 cells with Claudin -1 (CLN-1) and Occludin (OCC) siRNA knock downs

P. berghei Replication with CLN-1 and OCC knockdowns

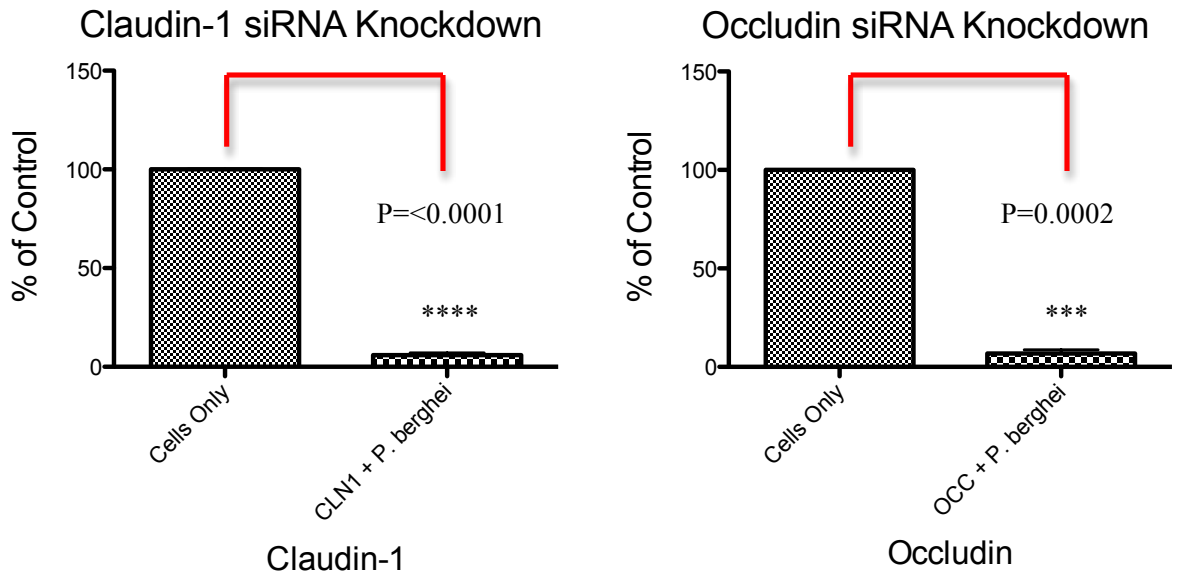
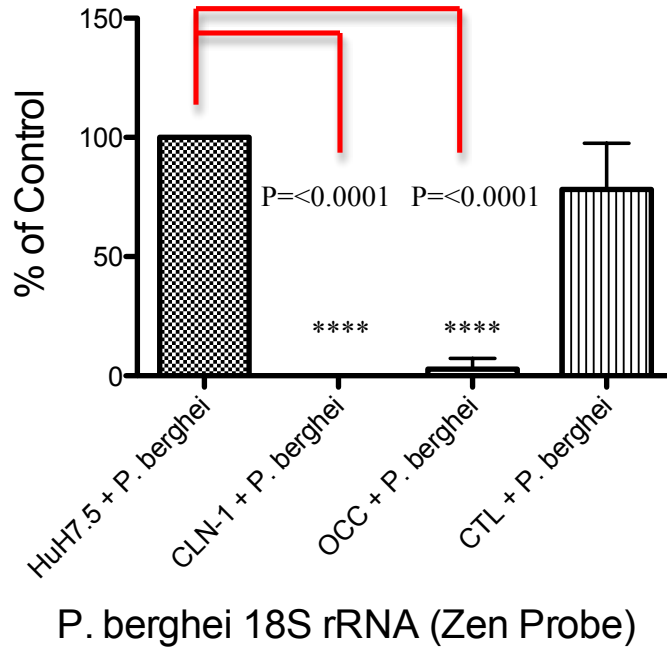
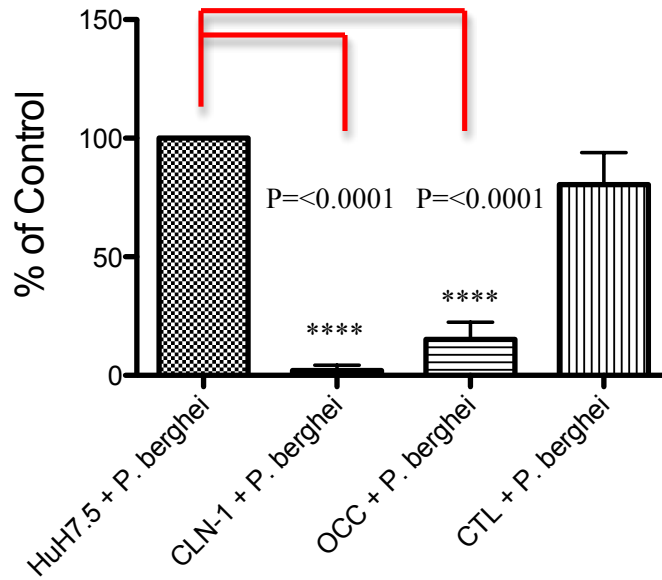


Figure 14. Experiment 2: Quantitative RT-PCR results for *P. berghei* replication in HuH7.5 cells with Claudin -1 (CLN-1) and Occludin (OCC) siRNA knock downs

P. berghei Replication with CLN-1 and OCC knockdowns



P. berghei 18S rRNA (Zen Probe)

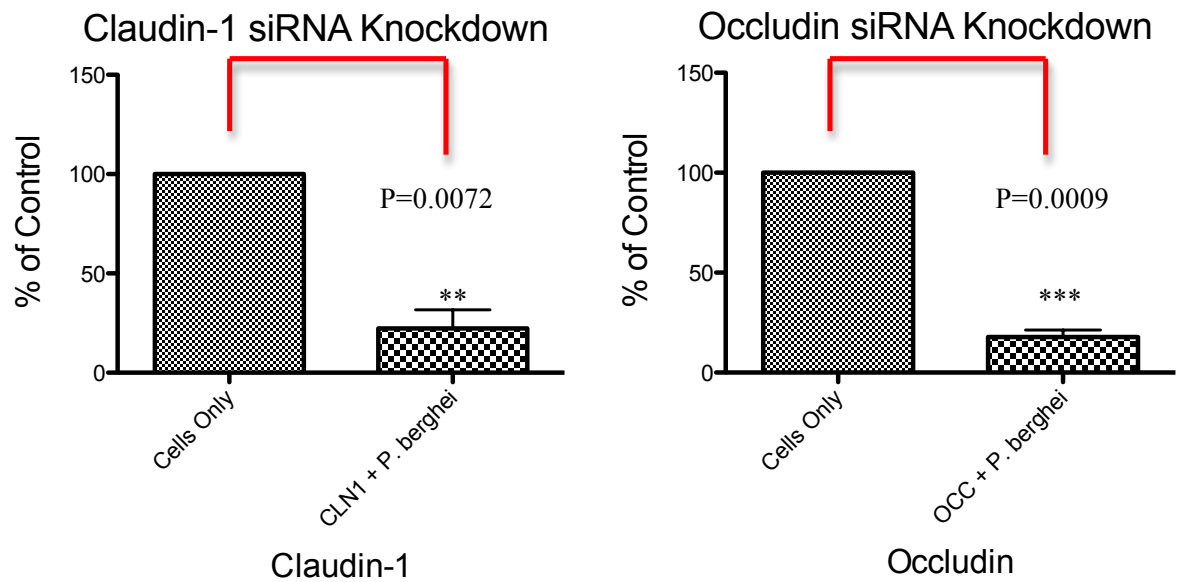


Figure 15. Experiment 3: Quantitative RT-PCR results for *P. berghei* replication in HuH7.5 cells with Claudin -1 (CLN-1) and Occludin (OCC) siRNA knock downs

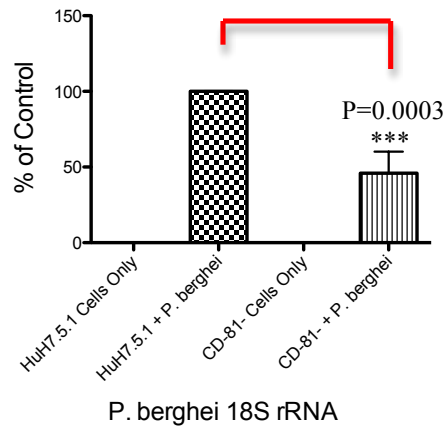
4.2.2 Examine *Plasmodium berghei* replication when infecting CD-81 deficient cells

Most *Plasmodium* species require CD-81 to enter hepatocytes; however, it has been shown that *Plasmodium berghei* utilizes other means to enter cells. Conversely, even though *P. berghei* can still enter cells without the use of CD-81, there is a decrease in the replication of the parasite during this stage of its life cycle. In order to further support this idea, *P. berghei* infection was examined in CD-81 deficient cells derived from HuH7.5.1 hepatoma cells.

Following infection, qRT-PCR was used to analyze the data (Fig 16). After statistical analysis (unpaired, two-sided t-test), it was concluded that *P. berghei* replication was not completely inhibited in CD-81 deficient cells; however, replication significantly decreased when infecting CD-81 deficient cells as shown in Figure 16 (P-value = 0.0030, 0.0161, 0.0064). Error bars represent sample standard deviation.

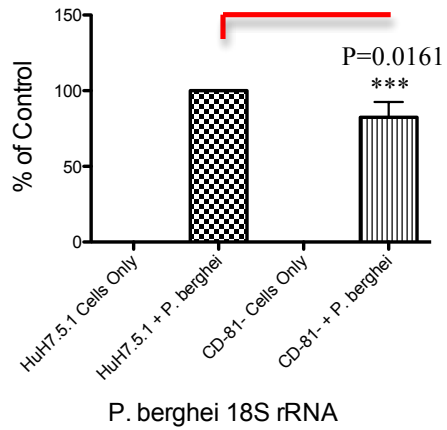
A. Experiment #1

P. berghei Replication in CD-81 Deficient Cells



B. Experiment #2

P. berghei Replication in CD-81 Deficient Cells



C. Experiment #3

P. berghei Replication in CD-81 Deficient Cells

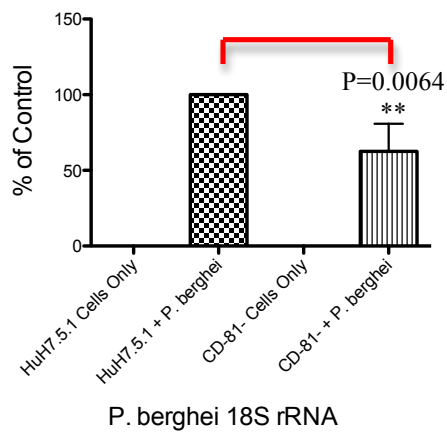


Figure 16. Quantitative RT-PCR results for *P. berghei* replication in CD-81 deficient cells

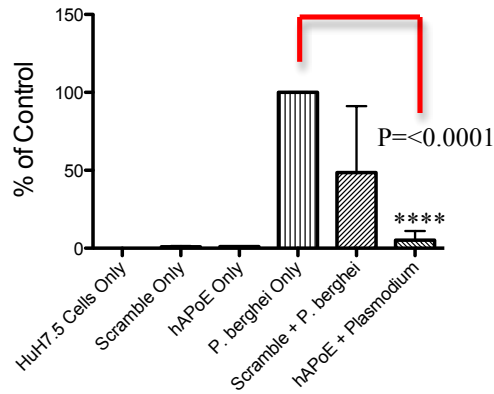
4.2.3 Examining *Plasmodium berghei* replication with ApoE peptide inhibition

Apolipoprotein E has recently been shown to be a key culprit in hepatitis C virus entry and, ultimately, replication within liver cells. To determine whether *Plasmodium berghei* utilize host factor ApoE upon entry into hepatocytes, human ApoE peptide was used to inhibit possible parasitic attachment and entry into the cell. HuH7.5 cells only were used as a negative control for the experiment, while a “scramble” peptide, known to have a random sequence that will cause no inhibition of entry, was used as a positive control.

Figure 17 shows that when human apoE peptide was introduced to HuH7.5 cells simultaneously with *P. berghei*, *P. berghei* replication significantly decreased (P-value = <0.0001, <0.0001, 0.0010). Data was statistically analyzed via student’s t-test.

A. Experiment #1

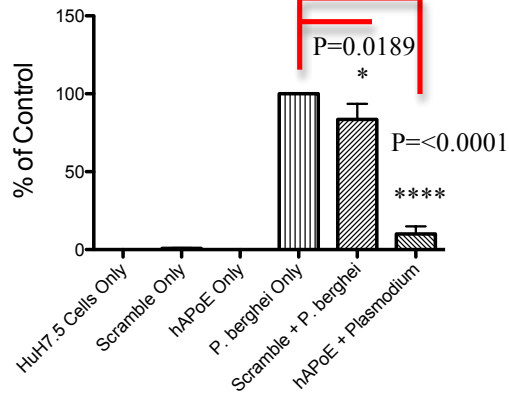
P.berghei Replication with ApoE peptide inhibition



P. berghei 18S rRNA (Zen Probe)

B. Experiment #2

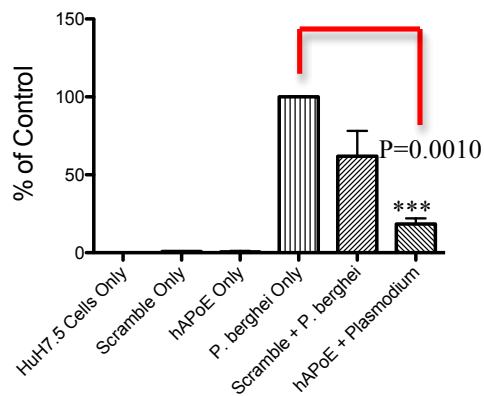
P.berghei Replication with ApoE peptide inhibition



P. berghei 18S rRNA (Zen Probe)

C. Experiment #3

P.berghei Replication with ApoE peptide inhibition



P. berghei 18S rRNA (Zen Probe)

Figure 17. Quantitative RT-PCR results for *P. berghei* replication with ApoE peptide inhibition

5.0 DISCUSSION

5.1 BRIEF OVERVIEW OF CURRENT STUDY

Hepatitis C and malaria are two diseases affecting millions worldwide. While there are no current epidemiological studies examining co-infection of these two diseases, it is plausible due to their endemic overlap. Furthermore, both pathogens, hepatitis C virus and *Plasmodium spp.*, utilize the liver as part of their life cycle to complete development. In addition, both hepatitis C virus and *Plasmodium spp.* rely on four host entry factors for attachment and entry into host hepatocytes: HSPGs, CD-81, SR-B1, and apoE.

In this study, we wished to examine each pathogen and their affects on one another when co-infected in various hepatoma cell lines. Additionally, we wanted to determine if both hepatitis c virus and *P. berghei* were able to co-localize in the same cell.

Since HCV and its entry into the host's hepatocytes has been largely studied, it was important to focus on *P. berghei* entry into the cell. Given that HCV and *Plasmodium spp.* have been shown to utilize some of the same host factors, examining other host factors HCV uses, such as claudin-1 and occludin, was necessary.

5.2 SIGNIFICANCE OF RESULTS

5.2.1 Developing an *in vitro* model to examine HCV and *Plasmodium* co-infection

5.2.1.1 Determine if co-infection in hepatocytes affects the development of either pathogen

After observing three independent experiments, the results seemed to be extremely varied, there were similar consistencies and trends in each individual cell line and trends among experiments for each cell line. Figure 8 shows HCV consistently decreases when *P. berghei* is present in the same sample, while *P. berghei* consistently increases when co-infected with HCV in HuH7 cells. Similar trends of HCV decreasing when co-infected with *P. berghei* were noted in Experiments 1 and 3 of Figure 7. Figure 8 also shows consistency of experiments taking place in HuH7.5 cells. Similarly, HCV decreased when in the presence of *P. berghei*, while *P. berghei* replication increased when co-infected with HCV. In Replicon cells (Fig 9), results show experiments 1 and 2 to be very consistent and generally, *P. berghei* decreases when HCV has already established itself within the cell. Even though there is variability in the samples, this variability could be due to the variability of sporozoite infectivity.

Upon receiving *P. berghei* infected mosquitoes from Dr. Photini at New York University, the amount of mosquitoes dissected and used for infection of hepatoma cells were different for every infection. In addition, the amount of sporozoites extracted from the salivary glands of the female mosquitoes varied with each batch of infected mosquitoes. While the total amount of sporozoites were counted before each infection and infected accordingly, the infectivity of the sporozoites could not be accessed until after infection through qRT-PCR and IFA. Even in optimal conditions during *in vitro* infection, *P. berghei* invasion rates have only been shown to be only 10-20% at the highest infectivity rate [41]. This may be due to the fact that there are

merely a small amount of infectious sporozoites within the salivary gland that are capable of invading host liver cells [41]. Furthermore, after entering the host during a female mosquito's blood meal, the sporozoites migrate through Kupffer cells of the liver and ultimately, transversing and damaging hepatocytes along the way until it remains within a suitable hepatocytes for further development [41]. The wounding and damaging of hepatocytes by the parasite may cause a decrease in the HCV that is able to develop within the hepatoma cells and may attribute to the decrease in replication as seen in our qRT-PCR results (Figures 7-9 and 11).

Furthermore, it was clearly seen that both *P. berghei* and HCV were more permissive to HuH7.5 and replicon cell lines. Once again, this may be due to the fact that a mutation in the RIG-1 gene deems it non-functional, and therefore, there is no cellular innate immune response. Immunofluorescent data from Figure 10 and Figure 12, show that more parasites and viruses can develop within these hepatocytes. In addition, HuH7 had the largest parasites when compared to the sizes of parasites in the other cell lines, suggesting that there is a difference in permissiveness for invasion (the amount of parasites that enter and develop) versus parasite development and replication (more parasite replication produces larger parasite schizonts).

5.2.1.2 Determine if pathogens are able to infect the same cell

After examining both pathogens and their affects on each other's development, it was important to determine if both HCV and *P. berghei* were able to infect and co-localize in the same cell. Four cell lines with co-infections of HCV and *P. berghei* were examined and compared to one another. It was evident from Figure 11 that HCV and *P. berghei* co-localization occurred in all cell lines with the exception of HuH7. This may be due to the lack of permissivity for HCV infection and since there seems to be a decrease in HCV when co-infected with *P. berghei*, it is plausible that HCV was not seen due to a lower infectivity rate. As stated before, *P. berghei* and

JHF-1 HCV were infected simultaneously. In general, *P. berghei* develops rather quickly *in vitro* by ultimately developing into merozoites and bursting the hepatocyte at approximately 63-64 h p.i.; while, HCV infection performed *in vitro* has been seen as early as 24 h p.i. [42]. It is possible that HCV infection and development occurred in most of the liver cells during our experiment, but was disrupted by parasitic development in the same cells. As before, parasites wound cells until finding a suitable hepatocyte for development; therefore, it is plausible that HCV replication decreased when co-infected with *P. berghei* because the parasites had wounded many of the cells infected with HCV.

Since the hepatoma cell's innate immune response to pathogens was inhibited by a point mutation in the RIG-1 gene, the permissivity of the cell line to invading pathogens increased and was noted following IFA and counting of parasites. HuH7 hepatoma cells have been utilized for *Plasmodium spp.* infections in the past, but it was clear that HuH7.5 hepatoma cells allowed for more *P. berghei* infection and development and produced similar sized parasites within each experiment. For this reason, HuH7.5 cells were utilized for the remainder of experiments. An increase in parasitic invasion and development was most likely due to the permissivity of the cell line.

5.2.2 Determine if *P. berghei* utilizes common host factors known to aid in HCV attachment and entrance into the cell

5.2.2.1 Examining *Plasmodium berghei* replication during Claudin-1 and Occludin siRNA knockdowns

In order to examine whether claudin-1 and occludin aid in *P. berghei* attachment and entrance into host hepatocytes, a transfection with claudin-1 and occludin siRNA was performed 48 hours prior to infection with *P. berghei* sporozoites. After performing qRT-PCR and statistical analysis, it is easy to conclude that when claudin-1 and occludin were knocked down, *P. berghei* replication decreased significantly; therefore, it is plausible that claudin-1 and occludin are utilized by *Plasmodium* when entering host hepatocytes. Hence, *P. berghei* development was hindered when claudin-1 and occludin do not aid in parasitic entrance into liver cells.

Both claudin-1 and occludin are host proteins located at the tight junctions between liver cells and have been shown to play a key role in regulating paracellular permeability and cell adhesion, particularly through phosphorylation and dephosphorylation [43,44]. It is plausible that following the activation of CSP and initiation of parasitic invasion that *Plasmodium spp.* causes a phosphorylation or dephosphorylation of these tight protein junctions in order to gain entrance into host liver cells. Like HCV, this phosphorylation of claudin-1 and occludin could play a similar role in facilitating *Plasmodium spp.* entry into host hepatocytes and ultimately, modify the development of the parasite within these cells.

While it is difficult to say whether these two entry factors are necessary for *P. berghei* entry, it is known that they are necessary for HCV entry. If both were needed, for *P. berghei* entry, it would make sense that the parasite and the virus would compete in order to enter the host hepatocytes; however, *P. berghei* replication seems to increase when co-infected with HCV,

indicating that the theory of competition may not hold true. At the same time, the parasite and virus could compete for claudin-1 and occludin host entry factors, explaining the decrease in HCV replication during co-infection. In order to test this theory, HCV could be added in a timely manner prior to infection with *P. berghei* and vice versa to ensure that each pathogen has ample time to enter the cell without having to compete. Quantitative RT-PCR analysis can be performed to determine the parasite and virus quantity, and ultimately, to decide whether the two pathogens compete for entry factors when co-infected.

5.2.2.2 Examine *Plasmodium berghei* replication when infecting CD-81 deficient cells

It was mentioned that *P. berghei* does not require CD-81 to enter and infect hepatocytes in Silvie, et al. (2007); however, it was important to perform our own experiment surrounding this conclusion made by Silvie, et al (2007) [23]. CD-81 deficient cells provided by Dr. Tianyi Wang served as a model for this experiment with HuH7.5.1 cells as a control, since the CD-81 deficient cells were derived from these cells. In agreement with Silvie, et al. (2007), in the absence of CD-81, *P. berghei* development was significantly hindered; however, it was still able to replicate. In conclusion, unlike HCV where CD-81 is required for entry into hepatocytes, it is not a requirement for *P. berghei*'s entry. It is probable that *P. berghei* utilizes many different pathways in order to infect cells, hence the decrease in *P. berghei* replication and not the complete inhibition of parasitic infection. It is important to note that *P. berghei* is a “promiscuous” species of rodent *Plasmodium*, meaning it is able to easily infect many different cell lines using different pathways. Other species of *Plasmodium* require CD-81 for entry into host hepatocytes. However, in this case, *P. berghei* does not require host entry factor CD-81 for entry into the cells simply because development is not hindered to its maximum potential.

5.2.2.3 Examining *Plasmodium berghei* replication with ApoE peptide inhibition

Apolipoprotein E has recently been shown to be a key culprit in hepatitis C virus entry and, ultimately, replication within liver cells. To determine whether *Plasmodium berghei* utilize host protein apoE upon entry into hepatocytes, human apoE peptide was used to inhibit possible parasitic attachment and entry into the cell. This host protein is responsible for combining with lipid in the body to form lipoproteins. Referred to as a very low density lipoprotein (VLDL), apoE is accountable for removing excess cholesterol from the blood and carrying it to the liver for processing, hence, it is necessary for facilitating lipid import into liver cells [45]. All in all, pathogens, like HCV and *Plasmodium spp.*, could utilize apoE as a “mask” in order to enter and invade host hepatocytes.

The apoE peptide, hEP, produced by Dr. Tianyi Wang as described in Lui, et al (2011), was designed to contain the LDLR binding region, the two heparin binding regions, and the major lipid binding region of apoE [32]. The peptide competes with virion-associated apoE for cellular receptors or lipids, hence decreasing the ability of virus attachment to hepatocytes [32]. Like HCV, it is reasonable that *Plasmodium spp.* utilize apoE in the same manner as HCV, by containing apoE protein that is associated to the host cell surface, which may initiate parasite entry into the liver cell. It was shown in Figure 17 that when apoE peptide was introduced simultaneously with *P. berghei* sporozoites, apoE significantly inhibited parasite entry into the liver cells.

Not only is apoE important for HCV entrance into host hepatocytes, it has also been shown to play an important role in successful virion assembly [31]. While HCV virions generally display heterogeneous densities, low-density HCV virions were rich in apoE protein

[31]. Following a knock down using apoE siRNA, Chang, et al. (2007) discovered that when apoE was not present, virion production intracellularly as well as virion secretion was significantly reduced [31]. Hence, it was concluded that apoE is required for HCV infectivity as well as production.

In addition, apoE within *Plasmodium spp.* sporozoites serves as a receptor for host HSPGs, which ultimately trigger a cascade of parasite CSP and, ultimately, parasite invasion [46]. While sporozoite entry utilizing apoE protein on the parasite has been examined, apoE's role in parasitic development has not. It is plausible that like HCV, *Plasmodium spp.* require apoE to develop into infectious parasites and continue their life cycle. While our results showed a significant decrease in *P. berghei* development, apoE could be required for infectious parasite maturity as well as entry into host hepatocytes. To further examine this, apoE peptide could be added after sporozoite entry has occurred to show apoE's affects on *P. berghei*'s development within the hepatocytes.

5.3 RELEVANCE OF STUDY

While not epidemiologically studied, given the epidemic overlap of hepatitis c and malaria in particular areas in the world, it is plausible that co-infections of these two diseases occur. In that case, one pathogen may cause an increase or decrease in the severity of the other and vice versa. Furthermore, while HCV attachment and entry have been heavily studied due to its increasing prevalence worldwide, *Plasmodium* entry into host hepatocytes is just starting to be examined. Due to the increase in resistance to anti-viral and anti-malarial treatments, it is critical to

investigate all possible routes of entry both pathogens utilize to enter the liver and develop in order to ultimately target the pathogens' means of access, development and spread of new virions and pathogen.

This study provides insight on new possible *Plasmodium* techniques for parasite entrance into host cells by taking another pathogen, in this case, HCV, whose known host factors for attachment and entry parallel what is known about *Plasmodium*. Since there are similarities of host factors for HCV and *Plasmodium* (HSPGs, CD-81, SR-B1, and apoE), well-known co-receptors for HCV attachment and entrance (claudin-1 and occludin), were utilized during this experiment and showed to obstruct *P. berghei* replication. This may provide insight to new anti-malaria drugs targeting parasite entrance into the cell.

Overall, HCV replication and development was reduced when co-infected with *P. berghei*. This may be due to a lower replication rate of the virus or competition for host entry factors upon entrance into the cell. Furthermore, it was seen that co-localization of both *P. berghei* and HCV in the same cell occurred in more permissive cell lines, primarily, those cell lines with a RIG-1 mutation. All in all, *P. berghei* replication decreased when host entry factors claudin-1, occludin, CD-81, and apoE were not available for use. Further studies could determine whether therapeutic inhibition of such factors could reduce malaria development at the liver stage, decreasing the amount of blood-stage parasites that develop, and in the end, reduce transmission from host to mosquito and back to host.

5.4 FUTURE DIRECTIONS

While evidence of each pathogen affecting the development of the other was quite unclear and variable, these pathogens have the capability to enter and develop in the liver simultaneously.

Future studies may also solidify the evidence that *P. berghei* development is significantly deterred when it cannot utilize co-receptors claudin-1 and occludin as a means of entry into hepatocytes. It would also be beneficial to examine HCV and *Plasmodium falciparum*, a human parasite, and the effects each pathogen have on one another.

Studies in the future may also look in-depth into the mechanisms by which *Plasmodium spp.* utilize these particular host factors and, thus, provide useful information to help produce new anti-malaria drugs to treat malaria in endemic areas worldwide. With this additional research and therapeutic intervention at the early stages of the *Plasmodium* life cycle, malaria transmission could be slowed or possibly eradicated in the near future.

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