AN ABSTRACT OF THE DISSERTATION OF

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Humans and viral disease are inextricably intertwined. Viral disease plays an immeasurable role in human life, from the disease and economic burden associated with every facet of contending with human viral disease, to managing the consequences of viral disease in organisms important to our food supply, economy, and entertainment. The studies within this dissertation encompass crucial areas of viral research: host-pathogen interactions and diagnostics. Chapters 2 and 3 of this dissertation describe both the study and manipulation of viral-pathogen interactions. The next two chapters describe the application of recombinase polymerase amplification (RPA) for the detection of two prominent viral pathogens in need of improved diagnostic strategies.

In Chapter 2, a host-viral pathogen interaction was exploited in an effort to develop a method for increasing influenza virus in the context of cell culture-based viral propagation for vaccine production purposes. PPMO antisense technology was employed to target and suppress the expression of the host gene Interferon alpha (IFNα), which is mainly involved in innate immune response against viral infection, in chicken embryo fibroblast (DF-1) cells. Suppression of IFNα by PPMO resulted in significantly reduced levels of IFNα protein in treated wells measured by ELISA and was shown to not have any cytotoxicity to DF-1 cells at the effective concentrations tested. Treatment of the self-directing PPMO increased the ability of the influenza virus to replicate in DF-1 cells. Over a three-fold increase in viral production was observed in PPMO treated wells compared to those of untreated controls, which was observed to be independent of the initial viral input. Our results indicate that the use of PPMOs to target host protein expression can result in increased production of influenza virus; a technology that could be used on its own for improvement of vaccine production strategies or as a screening tool for subsequent permanent alterations in cell culture lines that would have similarly increased influenza virus production.

In Chapter 3, host-viral pathogen interactions were examined in an attempt to understand an aspect of the host response to Respiratory Syncytial Virus (RSV) infection. The host gene, Myeloid Cell Leukemia 1 (Mcl-1), is upregulated early in RSV infection and is thought to have anti-apoptotic function. Mcl-1 knockout and wild type (WT) mouse embryonic fibroblast (MEF) cells were used to characterize the viral response to the absence of the host protein Mcl-1. The lack of Mcl-1 caused MEF cells to become highly permissive to RSV infection and resulted in extremely high levels of RSV compared to viral replication in WT MEF cells. Mcl-1 knockout cells also exhibited uncharacteristic morphology during RSV infection with increased and enlarged syncytial formation. Interestingly, apoptosis, which Mcl-1 helps regulate, was not induced in knockout cells until late in infection. The work

presented in Chapter 3 provides evidence that Mcl-1 upregulation in RSV infection would not be beneficial to the virus, rather Mcl-1 upregulation is most likely an antiviral strategy and suggests a possible function for Mcl-1 separate of apoptosis regulation.

In Chapter 4, we have developed a quick, sensitive, and adaptable recombinase polymerase amplification (RPA) diagnostic assay to detect a significant human pathogen, dengue virus (DENV). Dengue is considered the most important arbovirus worldwide and the World Health Organization has listed improved diagnosis as a key step in the fight to reduce the burden of DENV. We demonstrate that our DENV2 specific RT-RPA assay is sensitive and specific, as it is able to amplify DENV2 with as little as 50 copies per reaction within 20 minutes at a constant temperature, and was able to amplify both laboratory and clinical isolates/strains. Our results provide justification for future development of RPA as a diagnostic strategy for detection of DENV in a clinical setting at point-of-care, thus eliminating the need for a costly thermocycler.

In Chapter 5, RPA is applied to the herpes virus, cyprinid herpes virus 3 (CyHV-3). This DNA virus has had a considerable impact on koi and common carp that leads to devastating economic losses to both fishery and koi hobbyist. One problem with current diagnostics is the inability to reliably detect latently infected fish, capable of acting as carriers to nascent fish populations. The RPA assay to detect CyHV-3 was specific and sensitive, yielding results in approximately 20 minutes, and was able to detect the virus in latently infected koi more efficiently than a real-time PCR assay, when directly compared. RPA products were detected by a simple colorimetric lateral flow assay that could allow for detection outside of the diagnostic lab, allowing for sensitive and accurate surveillance and early diagnosis of CyHV-3 in the laboratory and field.

Overall, the studies herein provide valuable knowledge about viral diseases. The data collected provides insights into the characterization of host-

pathogen interactions of RSV. These insights are informative for the disease pathogenesis of this significant pathogen but may also apply to closely related viruses. In addition, a methodology is described in a new format that could prove to be valuable for influenza vaccine prevention strategies, as well as for any vaccine produced in cell culture. This work also describes the application of an isothermal amplification strategy for detection of two viral diseases that are in desperate need of improved diagnostics. RPA as a diagnostic tool is easily adaptable and can improve the speed, sensitivity, and resource consumption of viral diagnostics leading to the ability to detect viral disease at point-of-care or in low resource settings.

Host-pathogen Interactions of Respiratory RNA Viruses and the Application of Recombinase Polymerase Amplification for Viral Diagnostics

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Meagan A. Prescott, Author

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Chapter 5: Aimee Reed and Ling Jin provided koi white blood samples and contributed to experimental design and manuscript preparation. Aimee Reed conducted real-time PCR assays and assisted in additional data collection.

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Chapter 1. Introduction and Background

Host-pathogen interactions of respiratory RNA viruses

Influenza virus

General

Influenza virus is an enveloped, segmented, single stranded, negative sense RNA virus belonging to the *Orthomyxoviridae* family (Samji 2009). Influenza A viruses cause yearly seasonal epidemics, and are responsible for the periodic pandemics associated with high mortality recorded throughout history, most notably being the 1918 pandemic which took ~40 million lives worldwide (Krammer and Palese 2015). Clinical presentations of influenza vary, but the classical influenza is characterized as a febrile illness of both the upper and lower respiratory tract that is usually self-limited but can result in serious complications, especially in high risk groups such as young children and the elderly. In fact, during recent flu seasons, 80-90% of influenza related deaths in the United States have occurred in those 65 or older (CDC 2015b). Worldwide, seasonal influenza is estimated to affect 10% of adults and 30% of children resulting in millions of infections and thousands of deaths (WHO 2014b).

Viral genome and virion structure

The influenza genome is comprised of 10 genes located on eight segments that encode 11 proteins: hemagglutinin (HA), neuraminidase (NA), matrix 1 (M1), matrix 2 (M2), nucleoprotein (NP), non-structural protein 1 (NS1), non-structural protein 2 (NEP/NS2), polymerase acidic protein (PA), polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2) and polymerase basic protein 1 – F2 (PB1-F2) (summarized in Table 1.1) (Strauss and Strauss 2002, Samji 2009). The viral envelope is a lipid bilayer derived from the host's plasma membrane that contains the viral transmembrane proteins: HA, NA, and M2 (Nayak et al. 2009, Scheiffele et al. 1999, Zhang, Pekosz, and Lamb 2000). M1 forms the matrix which holds the viral ribonucleoproteins

(vRNPs), comprised of the RNA genome wrapped around NP with a small amount of NEP (Samji 2009). The final component of a mature virion is the polymerase complex (PB1, PB2, PA) which forms at one end of the vRNPs (Nayak et al. 2009).

Table 1.1. Summary of influenza gene segments, encoded proteins, and functions. Adapted from (Samji 2009, Strauss and Strauss 2002, Paterson and Fodor 2012).

Gene Segment	Protein Name	Function
1	Polymerase Basic 2 (PB2)	Cap snatching
2	Polymerase Basic 1 (PB1)	Major polymerase
	PB1-F2*	Induces apoptosis, role in pathogenesis
3	Polymerase Acidic (PA)	Endonuclease for cap snatching
4	Hemagglutinin (HA)	Receptor binding, membrane fusion
5	Nucleoprotein (NP)	Forms helical nucleocapsid, viral protein synthesis, nuclear export
6	Neuraminidase (NA)	Viral attachment and entry
7	Matrix 1 (M1)	Associates with cell membrane and NP, organizes directionality of vRNP transport
	Matrix 2 (M2)*	II+ Ion channel, unpackaging, polymerase activity, formation of virus particles
8	Non-structural (NS1)	Inhibits cellular mRNA transport, cap snatching, and IFN signaling
	Nuclear Export (NEP/NS2)	Nuclear export of nascent nucleocapsid, regulation of viral transcription, efficient release of nascent virions
* produced by splice variant or alternative reading frame		

Virus life cycle

The life cycle can be divided into several stages: entry into the host cell and vRNPs into the nucleus, transcription and replication of the viral genome, and

export of the vRNPs from the nucleus followed by assembly and budding of the mature virion. Influenza enters cells of the airway through receptor-mediated endocytosis by binding to sialic acid residues through the viral HA membrane glycoprotein (Nicholls et al. 2007, Takahashi et al. 2009, Shinya and Kawaoka 2006). As the endocytic compartment acidifies, the M2 protein opens an ion channel that acidifies the viral core and HA induces a conformational change that leads to eventual release of the vRNAs into the cytoplasm (Pinto and Lamb 2006, Samji 2009). Each of the viral proteins comprising the vRNPs (NP, PA, PB1, and PB2) has nuclear localization signals (NLSs) that facilitate the nuclear import of the vRNP by binding karyopherins (i.e. importin α and β) (Boulo et al. 2007, Samji 2009). The first step of viral replication in the nucleus is mRNA production in which viral proteins hijack the host's transcription machinery and utilizes a "cap-snatching" mechanism to prime viral transcripts for subsequent transcription (Reich et al. 2014, Guilligay et al. 2008, Dias et al. 2009). Viral transcripts are exported from the nucleus and after sufficient levels have accumulated, the viral polymerase, facilitated by NEP, initiates viral genome replication (Samji 2009). Following protein synthesis and genome replication, the viral components are exported from the nucleus, via a Crm1 dependent pathway mediated by NEP, NP, and M1 proteins, and packaged in the cell cytoplasm; nascent virus buds from the apical side of the host's polarized cells' plasma membrane by the sialic acid cleavage via the NA viral protein (Portela and Digard 2002, Robb et al. 2009, Nayak et al. 2009).

Genetic diversity

Influenza is classified into subtypes based on the HA and NA surface glycoproteins of which 16 HA and 9 NA varieties (characterized by antigenic properties) are currently recognized (Nandy et al. 2014). Within a given subtype, slight changes to the glycoproteins or other viral genes lead to

different strains of the virus. The driving force behind antigenic variation is the host immune response which targets the HA and NA proteins for humoral immunity and places a constant pressure on the virus to create and select for antigenically distinct viruses (Blackburne, Hay, and Goldstein 2008, Chen and Deng 2009).

Antigenic variation for the influenza virus occurs by two distinct and important processes: antigenic drift and antigenic shift. The more subtle and common method of variation in influenza is genetic drift and occurs when the viral polymerase produces mutations during the replication process (Nelson and Holmes 2007). These point mutations in the genetic code are frequent (but generally silent) and are typically responsible for the changes in antigenicity for circulating viruses (Asjö and Kruse 2007). Both cell-mediated and antibody responses develop after infection with influenza and antibody results in long lasting immunity against the infecting strain. However, the result of drift leads to the virus having just enough alterations to cause the seasonal epidemic infections, although they are less severe due to a small amount of preexisting immunity, and is the reason for the continual need for an updated influenza vaccine (Bedford et al. 2014, Bedford et al. 2015, Wikramaratna et al. 2013). Antigenic shift occurs when two separate influenza A viruses infect the same cell simultaneously and reassortment of the genomic segments can result in the formation of new HA and NA combinations that dramatically alter the antigenicity of the virus (Guan et al. 2010, Nelson and Holmes 2007). The production of novel influenza viruses by reassortment can lead to pandemic viral outbreaks and depending on the pathogenicity of the resulting virus, high morbidity and mortality may occur (Guan et al. 2010, Taubenberger and Morens 2006).

Vaccine

Influenza virus vaccines were first made in the 1940s and were generated in embryonated chicken eggs, the predominant technology still used today, which yields approximately one dose per egg (Krammer and Palese 2015, Buckland 2015). A number of vaccines are produced, including both inactivated influenza vaccines (IIVs) and live attenuated influenza vaccines (LAIVs), and are multivalent to protect against several different strains that are predicted to circulate in a given year (Krammer and Palese 2015). Antigenic drift of influenza virus makes constant reformulation of the vaccine necessary. Seasonal influenza vaccines are usually ineffective for protection against pandemic viruses, requiring new specific vaccines to be manufactured in the emergence of a pandemic virus (Krammer and Palese 2015). Vaccine production is time consuming, therefore pandemic specific vaccines could be administered too late. Also, egg systems do not always support the growth of pathogenic viruses (Krammer and Palese 2015, Buckland 2015). Producing influenza vaccines in cell culture has distinct advantages over the traditional system including: flexibility, faster production time, adequate availability of substrate to grow the virus, eliminated reliance on embryonated chicken eggs, maintains aseptic conditions, the virus remains antigenically unchanged, can grow viral strains not supported by egg-based methods, and elimination of egg proteins that cause allergic reactions (Reisinger et al. 2009, Mabrouk and Ellis 2002, Hütter et al. 2013, Buckland 2015). In recent years there has been recurrent annual influenza vaccine shortages in the United States, demonstrating the need for additional technologies to facilitate the production of the influenza vaccines (Reisinger et al. 2009).

Innate immune response

Viral infection is initially recognized by the host's innate immune system through the recognition of viral components by pattern-recognition receptors (PRRs) (Akira 2009, Medzhitov 2007, Ehrhardt et al. 2010). Three classes of PRRs have been shown to be involved in the recognition of viruses by the innate immune system: toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I) – like receptors (RLRs), and nucleotide oligomerization domain (NOD) – like receptors (NLRs) (Takeuchi and Akira 2009, Loo and Gale 2011). Recognition of viral components by TLRs and RLRs activate intracellular signaling cascades leading to the production of type I interferons (IFNs) and various cytokines and chemokines needed for activating effector-cells' function and to promote the development of the acquired immune response, important for viral clearance (Takeuchi and Akira 2009, Randall and Goodbourn 2008). TLRs 3, 7, 8, and 9 recognize viral nucleic acids in cytoplasmic vesicles while TLRs 2 and 4 recognize envelope proteins at the plasma membrane (Takeuchi and Akira 2009, Cervantes et al. 2012, Lee et al. 2014). Two RLRs recognize cytoplasmic viral RNA: RIG-I and MDA5 (Ehrhardt et al. 2010, Loo and Gale 2011, Liu et al. 2015).

IFNα and IFNβ are type I IFNs that are directly induced during viral infection, including influenza virus (Randall and Goodbourn 2008, Goodman et al. 2010). In influenza infection both TLR 7/8 and RIG-I have been demonstrated to play a critical role in the inducement of type I IFNs (Takeuchi and Akira 2009, Ichinohe 2010), but TLRs 3 and 10 have also been indicated to induce the innate immune response (Lee et al. 2014, Guillot et al. 2005). The type I IFN response modulates the antiviral immune response to influenza infection in a number of ways including: the upregulating Janus kinase (JAK)/ signal transducers and activators transcription (STAT) pathway (which leads a cascade of immune modulation), increases protein kinase R (PKR) activity,

induces orthomyxovirus resistance gene (Mx), GTPases and viperin, promotes maturation of DC cells, upregulates the activity of NK and CD8+ T cells, and promotes the division of memory CD8+ T cells (Ehrhardt et al. 2010, Randall and Goodbourn 2008).

PPMO

Peptide-conjugated morpholino oligomers (PPMO) are antisense compounds that mimic RNA to bind to and sterically block target mRNA, leading to effective, targeted gene knockdown. The PPMO is comprised of two covalently linked components: a phosphorodiamidate morpholino oligomer (PMO) and a cell penetrating peptide (CPP). The PMO portion is a synthetic nucleic acid analog, antisense to the target mRNA, allowing for the PPMO to sterically block the target gene and subsequent protein production (Summerton and Weller 1997). The PMO has key modifications (Figure 1.1) that make it nuclease resistant and metabolically stable (Hudziak et al. 1996, Youngblood et al. 2007). The CPP is an arginine rich peptide that autonomously facilitates PMO delivery into cells, without the need for transfection reagents or other manipulations (Abes et al. 2006, Moulton et al. 2004, Moulton and Moulton 2010).

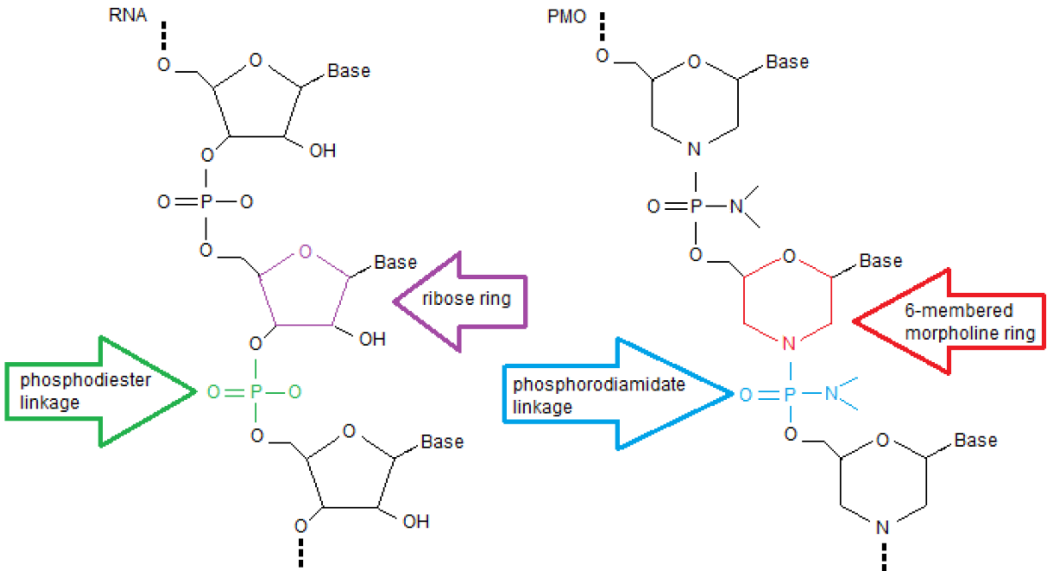


Figure 1.1. Structural comparison of RNA and PMO. Phosphorodiamidate morpholino oligomer (PMO) is modeled after RNA, but have key structural modifications. The PMO has the same nucleic acid bases, but those bases are bound to 6-membered morpholine rings instead of a ribose sugar and the rings are linked by a phosphorodiamidate linkage instead of a phosphodiester, as found in RNA. These modifications make the PMO uncharged and nuclease resistant; when attached to a cell penetrating peptide (now referred to as a PPMO), PMOs can be delivered autonomously.

Respiratory Syncytial Virus

General

Human Respiratory Syncytial Virus (hRSV, herein RSV) is an enveloped pneumovirus in the *Paramyxoviridae* family (Collins, Chanock, and Murphy 2001). The *Paramyxoviridae* family contains both the *Pneumovirinae* subfamily which RSV is a part, and the *Paramyxovirinae* subfamily that includes several well-known viruses: measles, mumps, and human parainfluenza virus. RSV was first isolated in 1956 from chimpanzees, then subsequently from human infants suffering from lower respiratory disease (Ogra 2004, Borchers et al. 2013). There is a single RSV serotype with two

subtypes (A and B), RSV A being more prevalent and slightly more pathogenic of the two (Borchers et al. 2013, Meng et al. 2014).

Viral genome and virion structure

The genome of RSV is a single strand of negative sensed RNA approximately 15 kb long that contains 10 genes encoding 11 proteins. Table 1.2 summarizes the RSV genes and encoded proteins with their primary or proposed functions. RSV proteins can be classified into two main groups: structural and nonstructural. The two nonstructural proteins (NS1 and NS2) are mainly thought to facilitate RSV replication by subversion of the host immune responses to infection (Bitko et al. 2007). The matrix (M) protein is a structural protein with several roles in infection and virion assembly, and the M2 encoded M2-2 protein is involved in the regulation of RNA synthesis (Ghildyal, Ho, and Jans 2006, Bermingham and Collins 1999); the remaining structural proteins make up the viral envelope and nucleocapsid. The envelope proteins are comprised of the glycoprotein (G), fusion glycoprotein (F), and the small hydrophobic protein (SH). These transmembrane envelope proteins function mainly in viral attachment and entry into the host cell (El Najjar, Schmitt, and Dutch 2014). The nucleocapsid is comprised of 4 proteins: nucleocapsid (N), phosphoprotein (P), large polymerase (L), and M2 encoded (M2-1) protein (Ghildyal, Ho, and Jans 2006).

Table 1.2. Summary of RSV gene names and encoded proteins. Adapted from (Ghildyal, Ho, and Jans 2006, Meng et al. 2014, Groskreutz et al. 2007, Espinoza et al. 2014)

Gene	Protein Name	Proposed function
NS1	Nonstructural 1	Host immune system interference: anti IFN type 1
NS2	Nonstructural 2	Host immune system interference: anti IFN type 1
N	Nucleoprotein	Formation of nucleocapsid, genome replication
P	Phosphoprotein	RNA Polymerase co-factor
M	Matrix	Host transcription inhibition, viral assembly and virion structure
SH	Small hydrophobic	Ion channel, host immune system regulation: anti-apoptosis
G	Glycoprotein	Viral attachment to host cells, host immune system modulation
F	Fusion glycoprotein	Viral entry, syncytia formation
M2	Matrix 2-1 (M2-1)	Transcription regulation
	Matrix 2-2 (M2-2)	Regulation of transcription and viral replication
L	Large (polymerase)	Viral RNA polymerase, inhibits apoptosis

Virus life cycle

Infection of RSV is initiated through the binding of the G protein to a cell surface receptor (most likely nucleolin) (Tayyari et al. 2011). Following the fusion of the viral membrane to a host cell, mediated by the F protein, the viral nucleocapsid is released into the cytoplasm where all subsequent steps of replication occur (El Najjar, Schmitt, and Dutch 2014). Transcription of the genome by the viral encoded polymerase utilizes a “stop-start” mechanism resulting in a gradient in the amount of mRNAs produced (Cowton, McGivern, and Fearns 2006). The order of genes is therefore synonymous with the

abundance of each viral protein produced starting at the 3’ end of the genome and diminishing towards the 5’ end. Replication of the genome is facilitated by the RNA polymerase after transcription has transpired and involves the production of a positive sense anti-genome to act as a template for synthesis of new negative sense genomic RNA that can then be used for additional replication, secondary transcription, or be incorporated into forming virus particles mediated by the M protein (El Najjar, Schmitt, and Dutch 2014). RSV proteins show preferential association with cholesterol rich lipid rafts and budding of the mature virion initiates from these sites (Malhotra et al. 2003).

Epidemiology

The global incidence and distribution of RSV make the virus a ubiquitous and notable pathogen. RSV is the primary cause of infant bronchiolitis, pediatric viral respiratory infection in the USA, and the most frequent cause of viral induced death in infants worldwide (Kling et al. 2014). Nair et al investigated the global burden of RSV in young children and reported nearly 200,000 deaths attributed to RSV infection within a single year, with a disparagingly high percentage (99%) occurring in developing countries (Nair et al. 2010). RSV is also a notable cause of adult morbidity; short-lived immunity results in the ability for recurrent RSV infection within a lifetime, and the elderly and those with high-risk conditions are susceptible to severe disease (Walsh and Falsey 2012). An estimated 17,000 adults die in the USA due to RSV infection annually (Walsh and Falsey 2012).

In tropical regions, the pattern of infection is unpredictable, but is typically fairly constant throughout the year; in temperate locales, there is a general seasonality to infectious cycles with an annual onset occurring in the late fall or early winter, peaking mid-December to early February, and finishing sometime in late spring (Borchers et al. 2013).

Generally, supportive care is the only form of management used in severe cases of RSV infection, and ribavirin, an antiviral therapy, is available but has questionable efficacy (Turner et al. 2014, Boeckh et al. 2007, Ventre and Randolph 2007). There is also a prophylactic treatment known as palivizumab that can be given to high risk patients (Turner et al. 2014, Hu and Robinson 2010). Although not yet available, several compounds are currently under development to treat RSV infection that include new monoclonal antibodies and several small molecule based inhibitors directed at fusion, attachment, and RNA synthesis (Mejias and Ramilo 2015, Chu and Englund 2013). Despite several trials, there is currently no vaccine available for RSV, although many avenues are being explored to develop a vaccination strategy including maternal immunizations for a protective effect in infants prior to when active immunizations would be protective (Meng et al. 2014).

Clinical disease, pathogenesis, and immunity

Clinical presentations of infection with RSV typically present as a mild upper respiratory tract illness, but in young children it can cause more severe lower respiratory tract infections like bronchiolitis or pneumonia (Turner et al. 2014). Severe RSV infection has also been linked to an increased risk for developing allergies and asthma (Sigurs et al. 2005, Sigurs et al. 2010). Pathogenesis is not well understood and it is unknown why a subset of otherwise healthy children develop severe disease when infected with RSV, but early innate immune response is thought to play a major role in the pathogenesis (Arruvito, Raiden, and Geffner 2015). In addition, some factors like low birth weight and cardiac disease are known risk factors in infants; impaired IFN-γ production has also been observed in infants that develop severe disease (Legg et al. 2003, Kling et al. 2014). RSV apically infects ciliated epithelial cells of the airways and airway mucus is a hallmark of RSV lower respiratory infection. This is a problem for infants with already small diameter airways,

but mechanistically, the inducement of mucus by RSV is not understood (Meng et al. 2014). Upon infection, epithelial cells produce a variety of cytokine and chemokines (MIP-1a, MCP-1, RANTES, IL-8, IFN- γ, IL-1β, IL-10, IL-12, IL-18, IL-6, TNFα, etc.) to activate the induction of host immune system and eventual clearance coordinated by CD8+ T cells (Borchers et al. 2013). Infection of RSV leads to the development of serum and mucosal IgM, IgG, and IgA antibodies (Ogra 2004). However, immunity to initial infection is not complete, and reinfection in early childhood and throughout one's lifetime is common (Meng et al. 2014).

Apoptosis

General

Apoptosis, or programmed cell death, is the physiological process by which unwanted or dying cells are packaged and labeled for rapid clearance. This involves a complex network of biochemical pathways and signaling cascades that ensure a homeostatic balance between cell proliferation and turnover in nearly all tissues (Chowdhury, Tharakan, and Bhat 2006, Elmore 2007). Apoptosis is marked by several features: the cellular cytoskeleton collapses, nuclear envelope disassembles, nuclear DNA fragments, and the cell surface is altered to cause the dying cell to be rapidly phagocytosed (Alberts et al. 2002). This results in the dead cell being recycled and the avoidance of the damaging consequences attributed to the alternate form of cell death, necrosis. Necrosis is a form of cell death that is generally characterized as an uncontrolled mode of cell death (although recent studies have indicated that defined signaling pathways can lead to necrosis) and necrotic cells often lead to chronic inflammatory immune reactions due to cell lysis (Vanlangenakker, Vanden Berghe, and Vandenabeele 2012). Apoptosis plays a critical role in several cell functions, including normal embryonic development, stress response, and immunity (Minet et al. 2006).

All pathways to apoptosis converge by the activation of caspases; a family of cysteine proteases that are activated by a process called the caspase cascade that leads to apoptosis (Ashkenazi 2008, Fan et al. 2005). Initial signaling stimulates the upstream initiator caspases (caspases 8-10) and recruit them to signaling complexes that promote the further cascade and activation of downstream caspases (caspases 3, 6, 7) by proteolytic processing and eventual cell death (Ashkenazi 2008). Two distinct, yet interlinked signaling pathways control the activation of apoptosis (brief overview of key steps of signaling cascades, Figure 1.2). Intracellular indicators activate the intrinsic pathway and extracellular signals, usually in the form of ligands, activate apoptosis through the extrinsic pathway. Pathways can be distinguished by whether they require activity by the BCL-2 family proteins and by the caspases crucial to the activation of apoptosis (Ashkenazi 2008, Youle and Strasser 2008).

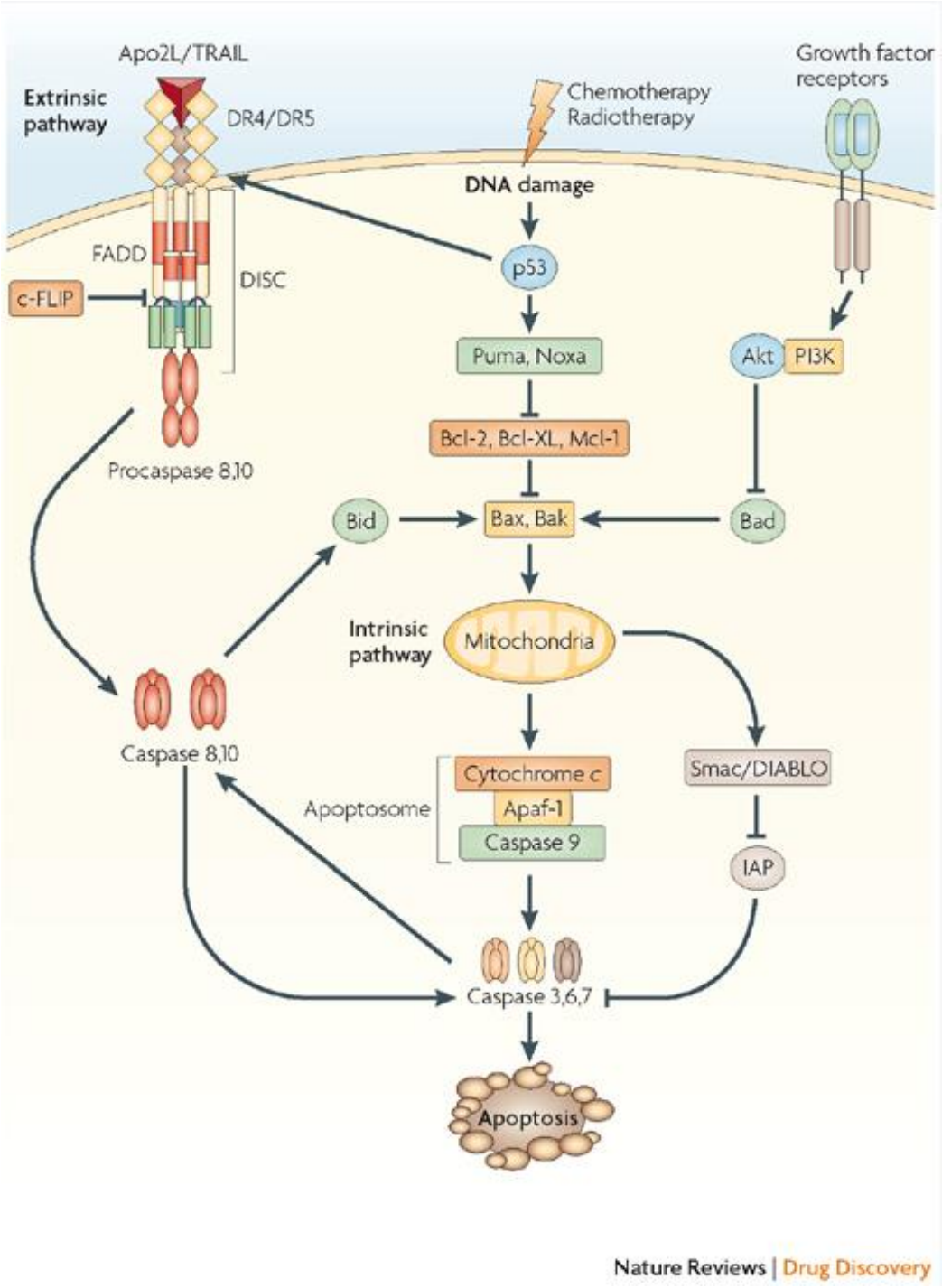


Figure 1.2. Key steps in the apoptotic signaling pathway. Image from (Ashkenazi 2008). The extrinsic pathway induces apoptosis through cell surface receptors such as tumor necrosis factor receptor-1 (TNFR1), Fas, or

ligands like Apo2L/TRAIL produced by cytotoxic immune cells. Alternatively, the intrinsic pathway is initially induced by internal signals like growth-factor deprivation, cellular stress, UV damage, or viral infection and acts through intracellular BCL-2 proteins. BCL-2 family members regulate the intrinsic pathway leading to apoptosis, but can also be involved in the extrinsic cascade through the modulator BID that engages the intrinsic pathway to further enhance apoptosis, initially induced through the extrinsic pathway.

Role of BCL-2 family

The BCL-2 protein family is essential for the activation and tight regulation of apoptosis through the intrinsic pathway, also called the BCL-2 regulated or mitochondrial pathway (Ashkenazi 2008, Youle and Strasser 2008, Burlacu 2003). There are a wide array of BCL-2 proteins with various structures but all members share homology with each other through a short BH3 motif (Youle and Strasser 2008). BCL-2 family proteins have opposing apoptotic activities that help modulate the apoptotic response by either inhibiting or promoting apoptosis. Pro-apoptotic members include BAX, BAK, BOK, BAD, BIK, BID, HRK, BIM, BMF, NOXA, and PUMA, whereas anti-apoptotic members include BCL-2, BCL-XL, BCL-W, Mcl-1, BCL-B, and A1 (Youle and Strasser 2008, Chipuk et al. 2010). A critical step in the intrinsic pathway is the permeabilization of the outer mitochondrial membrane (OMM), which results in the release of several apoptogenic factors (Neumann et al. 2015). BAX and BAK are key members of the BCL-2 family responsible for the OMM permeabilization and the subsequent release of apoptogenic molecules (cytochrome c, DIABLO) that lead to caspase activation; anti-apoptotic BCL-2 members antagonize this action by binding to these and other BCL-2 activators (Youle and Strasser 2008).

Role of Mcl-1

Myeloid cell leukemia factor-1 (Mcl-1), an anti-apoptotic regulator of apoptosis that antagonizes BAX and BAK, is unique in many ways to its family of

regulating proteins (Ola, Nawaz, and Ahsan 2011, Thomas, Lam, and Edwards 2010). Mcl-1 has several attributed functions and is structurally distinct from other BCL-2 members as it contains a long, unstructured, N-terminal domain followed by the BCL-2 core (Chipuk et al. 2010, Gui et al. 2015). In addition, Mcl-1 is the only anti-apoptotic BCL-2 protein with three BCL-2 homology domains, important for heterodimeric interaction among members of the BCL-2 family (Ola, Nawaz, and Ahsan 2011). Mcl-1 has been shown to be essential for embryonic development, survival of cell lineages (including lymphocytes, neutrophils, neurons, synovial fibroblasts, and hematopoietic stem cells), is involved in human cancers, and is associated with the survival and regulation of CD8+ T cell response to viral infection (Gui et al. 2015, Rinkenberger et al. 2000, Opferman et al. 2003, Perciavalle et al. 2012, Beroukhim et al. 2010, Fleischer et al. 2006, Liu et al. 2005). A list of factors have been shown to induce transcriptional upregulation of Mcl-1 including cytokines IL-3, 5, 6, growth factors, signal transducers and activators of transcription (STAT) family (Thomas, Lam, and Edwards 2010). Also, Mcl-1 has been shown to be upregulated in viral infection, including RSV (Lindemans et al. 2006, Zhong et al. 2012, Lupfer 2009).

Application of recombinase polymerase amplification

Dengue virus

General

Dengue virus (DENV) is a single stranded positive sense RNA virus of the *Flaviviridae* family. Members of the genus *Flavivirus*, of which DENV is apart, are the causative agents for significant disease worldwide including both tick-borne and some well-known mosquito-borne members: Yellow fever virus, Japanese encephalitis, and West Nile viruses (Kuno et al. 1998). Four antigenically distinct, but genetically related DENV serotypes (DENV1-4) circulate among humans and cause disease worldwide (Vasilakis et al. 2011).

Genome and infection cycle

DENVs positive sense genome is ~11 kb which encodes a single open reading frame (ORF), flanked by 5' and 3' untranslated regions (UTRs) (Gebhard, Filomatori, and Gamarnik 2011). The DENV genome encodes three structural proteins, capsid, pre-membrane (prM) and envelope (E), and at least seven non-structural proteins (NS), NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Gebhard, Filomatori, and Gamarnik 2011). Table 1.3 summarizes the DENV genome and protein functions.

Table 1.3. Summary of dengue virus genomic components with encoded proteins and functions. Components on genome 5' to 3' are in descending order. Adapted from (Gebhard, Filomatori, and Gamarnik 2011, Xie et al. 2013).

UTR/Gene	Protein Name	Proposed/ known function
5' UTR		Short stem-loop necessary for viral RNA synthesis, large stem-loop structure used as promoter for polymerase
C	Capsid	Virion structure, endosomal fusion, assembly
prM/M	Pre-membrane/ Membrane	Viral entry, mature virion structure
E	Envelope	Virion structure, viral entry, endosomal membrane fusion, assembly
NS1	NS1	Essential in RNA replication, facilitates immune complex formation
NS2A	NS2A	Hydrophobic membrane protein, scaffold for replication complex and important for pathogenesis and immune modulation
NS2B	NS2B	Co-factor for NS3
NS3	NS3	Viral serine protease/helicase, viral assembly
NS4A	NS4A	Hydrophobic membrane protein, scaffold for replication complex, induces membrane rearrangement

NS4B	NS4B	Hydrophobic membrane protein, scaffold for replication complex, co-locates with double-stranded RNA, suppresses interferon type 1 response
NS5	NS5	C-terminal two-thirds is the RNA-dependent RNA polymerase, N terminal one-third portion involved in viral cap formation and methylation
3' UTR		Viral replication

DENV virion is an enveloped particle with two structural envelope proteins: membrane (M) and E (Perera and Kuhn 2008). The envelope houses the ribonucleoprotein complex formed by the C protein bound to the viral genome (Freire et al. 2013, Kuhn et al. 2002).

Infection by DENV starts with attachment of the viral surface proteins to receptor molecules on the cell surface and subsequent internalization by receptor – mediated endocytosis. A specific receptor for DENV has not been identified, but several punitive cell surface components are implicated in attachment and entry of DENV which include: heparin sulfate (GAG), mannose receptor, DC-SIGN, HSP90/70, and TIM/TAM (Cruz-Oliveira et al. 2015). Following endosomal acidification, the viral envelope then fuses with the endosomal membrane allowing the viral genome to be released into the cytoplasm to serve as mRNA for protein synthesis (Gebhard, Filomatori, and Gamarnik 2011). Protein synthesis occurs at the endoplasmic reticulum (ER) and the resulting polyprotein is cleaved into mature proteins (Gebhard, Filomatori, and Gamarnik 2011). Following protein synthesis, the viral genome is replicated in vesicle packets (Gillespie et al. 2010). Assembly of nascent particles occurs in the ER and once packaged, virions are transported through the trans-Golgi network where virus maturation occurs (cellular protease cleaves prM generating M protein) followed by viral secretion (Cruz-Oliveira et al. 2015).

As a note, antibody dependent enhancement (ADE) during secondary dengue infection is thought to be mediated by Fcγ receptors (FcγR); antibodies produced during primary infection with DENV recognize and bind to a heterologous serotype upon subsequent infection, but instead of promoting viral neutralization and clearance, viral infectivity is enhanced when FcγR bearing cells internalize virus-containing immune-complexes (Cruz-Oliveira et al. 2015).

Clinical signs and disease management

DENV infection results in a wide range of clinical manifestations. Most cases are subclinical or result in the classical dengue fever (DF), characterized by fever, muscle and joint pain, and rash. However, a small portion of those infected will advance to more serve manifestations of disease: dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) characterized by sudden vascular permeability generated by cytokine release (Halstead 2007).

Infection by one serotype results in lifelong immunity to that serotype, but does not protect against subsequent infection of a different serotype. Successive secondary infections have been linked to increased risk of developing more severe disease (Vasilakis et al. 2011, Guzman, Alvarez, and Halstead 2013, Endy et al. 2004, Gibbons et al. 2007). A host of other factors implicated in increased risk of severe disease include: the genetic background of the individual (Perez et al. 2010, Nguyen et al. 2008, Chen et al. 2009, Alagarasu, Bachal, Tillu, et al. 2015, Alagarasu, Bachal, Memane, et al. 2015, Sa-Ngasang et al. 2014, Appanna et al. 2010, Chuansumrit et al. 2013, Stephens 2010), age (severe in young and old) and gender (female) (Guzmán et al. 1984, Anders et al. 2011, Aung et al. 2013, Huy et al. 2013, García-Rivera and Rigau-Pérez 2003, Pham et al. 2007, Guzmán et al. 2002, Lee et al. 2006), nutrition (Kalayanarooj and Nimmannitya 2005, Nguyen et

al. 2005), viral titer in infected individual (Vaughn et al. 2000, Libraty, Endy, et al. 2002, Libraty, Young, et al. 2002), and the strain of infecting virus (Vaughn et al. 2000, de Araújo et al. 2009, Fried et al. 2010, Thomas et al. 2008).

Currently, there is no effective antiviral agent to treat dengue, nor is there a licensed vaccine; treatment remains supportive (emphasis on fluid management) and prevention is limited to vector control (WHO 2009). In cases of severe disease, close observation is key with prompt fluid therapy vital in the management of disease, as well as blood transfusions, which can be lifesaving for those patients progressing to more severe symptoms (Simmons et al. 2012). A dengue vaccine appears to be very close as there are numerous dengue vaccine candidates being tested for safety and efficacy all in different stages of the testing pipeline from pre-clinical to advanced clinical development (Thomas and Rothman 2015). The most advanced dengue vaccine candidate is a chimeric vaccine called CYD-TDV and could be available as early as 2016, however this vaccine has shown mixed results for efficacy in clinical trials (Thomas and Rothman 2015, Simmons 2015).

Most developing countries have epidemics of febrile illnesses (measles, typhoid fever, SARS) that can be confused with DF which leads to unnecessary treatment and hospitalizations (Potts and Rothman 2008). Proper identification leading to avoidance of additional strain placed on health systems in these areas would be of economic benefit to already taxed systems.

Transmission

There are two ecologically and evolutionary distinct transmission cycles for dengue: a sylvatic cycle and a human cycle. The sylvatic cycle involves non-human primates and arboreal *Aedes* mosquitoes (Vasilakis et al. 2011). These sylvatic dengue species have the potential to shift from the animal reservoir and infect humans as sporadic zoonotic infections or become part of

the human cycle as a new or additional serotype (Vasilakis et al. 2011). In late 2013, media reported a fifth dengue serotype; following analysis it was shown that while distinct, this virus followed the sylvatic cycle and the human sample was a single infection from a zoonotic event that transferred the virus into a human host (Mustafa et al. 2015). Based on genetic studies the current serotypes are thought to have evolved from ancestral sylvatic progenitors that emerged separately into the human transmission cycle; the new serotype is a concern for future dengue related public health as there are very few adaptive barriers for the emergence of sylvatic DENV into human populations (Vasilakis et al. 2011, Wang et al. 2000).

Dengue is an arbovirus transmitted by mosquitos of the *Aedes* genus: *Aedes aegypti and Aedes albopictus (Simmons et al. 2012).* There is a five day period when a mosquito has large titers in its blood and can transfer the virus to a human host. After the virus has entered a mosquito who has taken a blood meal from a human host, an additional 8-12 days are needed before the virus can be transferred to another human (CDC 2014). Some cases have also shown organ transplant, blood transfusion, or vertical transmission (mother to fetus) as additional routes of transmission, but these types of transfer are considered to be rare (Arellanos-Soto et al. 2015, Stramer et al. 2012, Tambyah et al. 2008, Costa et al. 2015, Wiwanitkit 2010, Morgan-Ortiz et al. 2014). Seasonal patterns are common for endemic countries with low transmission season beginning in March and lasting until June and high transmission from August to November (CDC 2014). The distribution of DENV vectors has now been recorded on all continents; factors responsible for the rapid expansion of the mosquitoes' range are not fully understood, but climate factors as well as urbanization are major contributors (Kraemer et al. 2015, Aström et al. 2012, Brown et al. 2014).

Distribution

DENV remained geographically restricted until the middle of the 20th century when the *Aedes* mosquitoes were transported around the world facilitated by the increase in trade and human movement; now DENV is found worldwide and spreads anywhere the mosquito vectors can live and thrive – usually tropic or subtropic locales (Gubler 2006, Tatem, Hay, and Rogers 2006, Brown et al. 2014). Dengue is endemic in over 100 countries, where infections are common and occur every year, usually when mosquito populations are high after rainfall leads to optimal breading conditions (WHO 2012). Estimates of the global burden vary, but remain somewhere between 40% and 55% of the world's population live in areas where there is risk of dengue transmission, and the last five decades have seen an increase in dengue infection by 30-fold (Brady et al. 2012, Organization 2012). Yearly infections of DENV are predicted to be anywhere from 100-390 million cases (Bhatt et al. 2013, Beatty, Letson, and Margolis 2008). In the United States DENV infection is rare, usually acquired by travelers or immigrants, however, DENV has caused isolated outbreaks in the states: south Texas in 2005 (Ramos et al. 2008), Hawaii in 2001 (DENV1) (Effler et al. 2005), and southern Florida in 2009/2010 (Messenger et al. 2014, (CDC) 2010), as well as more sporadic infections in these same locales as well as several southern border states (Rawlings et al. 1998, CDC 2014, Waterman, Margolis, and Sejvar 2015, Añez and Rios 2013). Also of note, the U.S. territories (Puerto Rico, the U. S. Virgin Islands, Samoa, and Guam) are all endemic locations for DENV where island-wide epidemics, as well as constant yearly infections, have been well documented (CDC 2014, Añez and Rios 2013).

Diagnostic tests

Diagnosis of dengue can be established by a number of detection methods including detection of virus, nucleic acids, antigens or antibodies, or a

combination of these. Virus and viral nucleic acids can be detected up to 5 days after symptoms; following this time point, serology detecting antibodies must be employed. In general, dengue detection has been limited by restrictions of tests available; tests with high sensitivity and specificity require complex technologies or technical expertise, while tests that produce results quickly suffer from reduced sensitivity and specificity.

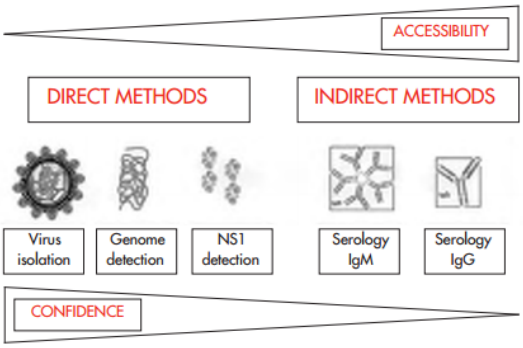
Virus isolation in cell culture is common for reference centers were samples are sent to be analyzed after infection has cleared, but are not generally employed in clinical settings due to the technical nature of culturing the virus. Detection of antigens by ELISA and dot blot assays to detect E/M proteins or NS-1 is used up to nine days after infection.

A primary infection of DENV results in IgM being produced first; 50% of patients have DENV specific IgM 3-5 days after symptom onset, 80% by day 5 and 99% by day 10 (WHO 2009). IgM levels generally peak at 2 weeks and decline to undetectable levels 2-3 months following infection. Anti-DENV serum IgG is detectable at low levels starting at the end of the first week of illness and slowly increases thereafter. During a secondary infection, antibody titers rise quickly and are dominated by IgG. An IgM/IgG antibody ratio is used to distinguish between primary and secondary infection (Falconar, de Plata, and Romero-Vivas 2006, Shu et al. 2003). Several serological tests are also available using ELISA to detect IgM (MAC-ELISA), IgG (IgG ELISA), and rarely used IgA. Serology tests have variable sensitivity and specificity, but only work well after the onset of symptoms making them inept for differential detection needed at point of care, early in infection.

Nucleic acid detection is generally an ideal method of detection that reduces time compared to viral isolation but allows for sensitive detection. PCR detection was the first wave of nucleic acid detection and generally exhibited good sensitivity with proper controls and good primer design. Therefore a

number of RT-PCR and real time RT-PCR assays have been utilized for dengue viral detection. The most commonly used in laboratories is the Lanciotti PCR assay, a nested PCR assay using universal dengue primers targeting the C/prM region of the genome followed by a nested PCR amplification that is serotype-specific (Lanciotti et al. 1992). Other RT-PCR assays developed have been: one-step multiplex RT-PCR (Harris et al. 1998), real-time fourplex RT-PCR (Johnson, Russell, and Lanciotti 2005), several commercially available kits (Simplexa, RealStar RT-PCR, Dengue virus general type real-time RT-PCR kit Liferiver, and Geno-Sen's dengue 1-4 real-time RT-PCR kit) (Najioullah, Viron, and Césaire 2014), and a real-time detection and typing RT-PCR assay developed by the CDC (Santiago et al. 2013). While PCR strategies have been considered to be the gold standard in Dengue detection, allowing for early detection of the virus, they require expensive technology and expertise that is not ideal for detection of dengue where early, quick, and easy to use diagnostics are needed.

Recently, isothermal amplification techniques have been designed and employed for many pathogens of interest due to their capacity for sensitive and quick amplification without the need for expensive thermal cycling instrumentation. Nucleic acid sequence based amplification (NASBA) (Wu et al. 2001) and reverse transcription loop mediated isothermal amplification (RT-LAMP) (Sahni et al. 2013, Teoh et al. 2013) have been described as an alternative method for DENV nucleic acid detection. Figure 1.3 summarizes the advantages and disadvantages of diagnostic methods used in clinical settings (WHO 2009).



Indications	Diagnostic Tests	Advantages	Limitations
Diagnosis of acute dengue infection	Nucleic acid detection	•Most sensitive and specific •Possible to identify serotype •Early appearance (pre-antibody), so opportunity to impact on patient management	•Potential false positive due to contamination •Expensive •Needs expertise and expensive laboratory equipment •Not possible to differentiate between primary and secondary infection
	Isolation in cell culture and identification using immuno-fluorescence	•Specific •Possible to identify serotype by using specific antibodies	•Need expertise and facility for cell culture and fluorescent microscopy •Takes more than 1 week •Not possible to differentiate between primary and secondary infection
	Antigen detection in clinical specimens	•Easy to perform •Opportunity for early diagnosis may impact on patient treatment	•Not as sensitive as virus isolation or RNA detection
	Serologic tests: IgM tests Seroconversion: 4-fold rise in HI or ELISA IgG titres between acute and convalescent samples	•Useful for confirmation of acute infection •Least expensive •Easy to perform •Can distinguish between primary and secondary infection	•May miss cases because IgM levels may be low or undetectable in some secondary infections •Need two samples •Delay in confirming diagnosis
Surveillance and outbreak identification; Monitor effectiveness of interventions	IgM detection Viral isolation and RNA detection	•Identify probable dengue cases •Easy to perform for case detection in sentinel laboratories •Confirm cases •Identify serotypes	•May miss cases because IgM levels may be low in secondary infections •Can be performed only in reference laboratories •Need acute samples

Figure 1.3. Summary of clinically available diagnostic tests. Adapted from figure/table within the WHO report for Dengue: Guidelines for diagnosis, treatment, and control (WHO 2009). Figure describes the advantages and limitations to currently utilized tests for dengue detection and compares accessibility and confidence.

Cyprinid herpesvirus type 3 (CyHV-3)

General and Importance

Cyprinid herpesvirus type 3 (CyHV-3) is newly emerging virus in the *Alloherpesviridae* family, infecting common and koi carp (Waltzek et al. 2009).

Since CyHV-3's emergence in the late 1990s, this highly contagious and pathogenic virus has caused severe economic losses worldwide for both common carp aquaculture as well as the ornamental koi industries. Common carp is one of the most economically valuable species in aquaculture ranked third in worldwide production with 3.8 million tons produced, representing 9.8% of the freshwater fish production and a US$5.2 billion industry (Boutier et al. 2015). The colorful, ornamental varieties (koi carp) are raised and housed for a number of purposes including hobbyist and competitive exhibitors which represent a significant source of additional economic revenue (Rakus et al. 2013).

Characterization

Herpesviruses infect a wide range of vertebrate and invertebrate hosts, but the host range of individual species is generally restricted (Rakus et al. 2013). The *Alloherpesviridae* family is newly designated to regroup herpesvirues infecting fish and amphibians that is divided into four genera: *Cyprinivirus, Ictalurivirus, Salmonivirus,* and *Batrachovirus* (Waltzek et al. 2009, Davison et al. 2013). The genus *Cyprinivirus* contains viruses with the largest Cyprinid herpesvirus genomes (248-295 kb) that are phylogenetically distinct from the other three genera that infect common carp (Cyprinid herpesvirus 1 and 3; CyHV1 and CyHV3), goldfish (Cyprinid herpesvirus 2; CyHV2), and freshwater eel (Anguilid herpesvirus 1; AngHV-1) (Rakus et al. 2013).

Both common carp (*Cyprinus carpip carpio*) and ornamental koi carp (*Cyprinus carpio koi*) are the primary hosts for the virus, but a number of hybrid crosses are also susceptible to infection (Dixon et al. 2009, Bergmann, Sadowski, et al. 2010). In addition, goldfish are susceptible to infection as the virus has been detected by real-time PCR in goldfish that cohabitated with CyHV-3 infected koi (El-Matbouli, Saleh, and Soliman 2007, Sadler, Marecaux, and Goodwin 2008). These goldfish did not exhibit any clinical

signs of infection, nor did they succumb to the otherwise highly lethal virus, suggesting goldfish are a carrier species (El-Matbouli and Soliman 2011). This is of particular importance as goldfish and koi often cohabitate, highlighting the implications for koi hobbyists and distributors in the health of their cyprinid fish populations.

CyHV-3 infection is highly contagious and extremely virulent with mortality rates that can reach from 80 to 100% in infected populations (Rakus et al. 2013, Hedrick et al. 2000, Bergmann, Sadowski, et al. 2010). Clinical disease has been seen as early as two days with mortality peaking on day 7-11 (Gilad et al. 2002). Most fish exhibit lethargy while others show neurologic symptoms, respiratory distress, necrotic gill tissue, and skin damage from excess mucus production and dermal ulceration as well as hemorrhaging of scales and fins (Grimmett et al. 2006, Gotesman et al. 2013). The virus replicates in gills, intestine, interstitial, liver, brain, and kidney tissues (Pikarsky et al. 2004). CyHV-3 has been demonstrated to be transmitted through water via the skin (Ronsmans et al. 2014). Environmentally, the temperature of the water is a key factor in the transmission of CyHV-3 (optimal growth at 15-25°C in cell lines); spring outbreaks are common due to the slight increase in temperature causing more permissive conditions (Yuasa, Ito, and Sano 2008, Uchii et al. 2014).

Viral latency is a hallmark of herpesvirus infection in which viral genome persist inside a host cell without the presence or production of nascent virions and restricted viral gene expression (Minarovits, Gonczol, and Valyi-Nagy 2007). Latency in CyHV-3 has been described with B lymphocytes identified as the main site for latently infected fish (Reed et al. 2014, Eide et al. 2011, St-Hilaire et al. 2005).

Genome and genotypes

The CyHV-3 genome is a linear, double stranded DNA molecule consisting of a central unique region flanked by two 22 kb repeat regions, and is the largest known herpesvirus to date at 295 kb (Aoki et al. 2007, Davison et al. 2013). The overall GC content is 59.2% and the genome has a predicted 156 open reading frames (ORF) (Aoki et al. 2007). Among the *Alloherpesviridae* family, 12 ORFs are conserved in all sequenced viruses, CyHV1-3 viruses each possess 120 orthologous ORFs, and CyHV-3 contains 21 unique ORFs (Davison et al. 2013).

CyHV-3 has at least three main genotypes: Asian strain originally isolated from Japan (CyHV-3 J) and two European strains originating from Israel (CyHV-3 I) and the United States (CyHV-3 U) (Kurita et al. 2009, Avarre et al. 2011). An additional lineage from Indonesia has also been proposed as an intermediate genetic lineage (Sunarto et al. 2011).

Epidemiology

CyHV-3 was first identified as the causative agent of disease from isolates obtained in the United States and Israel (Hedrick et al. 2000). Subsequent publications identified CyHV-3 from earlier isolates from England in 1996 and Germany in 1997 (Bretzinger et al. 1999, Haenen et al. 2004). Following these initial observations, CyHV-3 rapidly spread around the globe infecting aquaculture, wild, and koi carp populations and has been implicated in several mass mortality events around the globe (Choi et al. 2004, Grimmett et al. 2006, Garver et al. 2010, Marek et al. 2010, Avarre et al. 2012, Cheng et al. 2011, Dong et al. 2013, Bondad-Reantaso et al. 2005).

Diagnosis

Several methods have been employed to diagnose CyHV-3 infection. Real-time PCR strategies have been described the most due to the high sensitivity capable with genome amplification. Some of the first PCR assays were

described by Gilad et al (Gilad et al. 2002, Gilad et al. 2004). Since then, many assays have been developed that amplify CyHV-3 genome: PCR targeting DNA polymerase and major envelope protein genes (Ishioka et al. 2005), targeting the TK gene (Bercovier et al. 2005), mRNA specific PCR (Yuasa et al. 2012), and nested and semi-nested PCR assays (Bergmann, Riechardt, et al. 2010, El-Matbouli, Rucker, and Soliman 2007, Bercovier et al. 2005). In addition to these PCR based assays, an enzyme-linked immunosorbent assay (ELISA) was developed (St-Hilaire et al. 2009) as well as immunohistochemistry assays using monoclonal antibodies against CyHV-3 viral proteins for the detection of virus in infected tissues (Aoki et al. 2011, Tu et al. 2014). Detection in tissues has also been described by hybridization assays (Monaghan et al. 2015, Saleh and El-Matbouli 2015). The isothermal amplification of the viral genome has also been described using loop-mediated isothermal amplification (Gunimaladevi et al. 2004, Soliman and El-Matbouli 2005, 2010, Yoshino et al. 2009). Isothermal amplification techniques are used in an attempt to get rapid diagnosis that is sensitive and yet has the capacity to be performed tank side.

Isothermal nucleic acid amplification

General

Nucleic acid amplification is a fundamental process for both biotechnology and molecular biology and has been utilized in nearly every field of biology since its advent. PCR was the first amplification method developed and has been the predominate method of choice since its inception; the introduction of PCR has radically transformed biological sciences (Garibyan and Avashia 2013, Fakruddin et al. 2013). PCR has drawbacks that make its use unappealing for diagnostic purposes, especially as the need for rapid, easy to use, point-of-care diagnostics for resource limited settings increases (Deng and Gao 2015); PCR requires expensive, sophisticated, and bulky machinery,

especially for real-time application. In addition, PCR requires thermocycling to amplify products which increases reaction time, creates the need for primers whose melting temperatures are similar, and has high energy consumption. These limitations as well as others led to the development of isothermal nucleic acid amplification technologies which circumvent some of the limitations of PCR (Fakruddin et al. 2013, Deng and Gao 2015). Several of these technologies have been exploited for detection purposes in an effort to improve upon PCR methods and have been shown to outperform their PCR-based counterparts (Deng and Gao 2015). A number of isothermal amplification strategies have been described since the early 90s and vary in several ways: reaction temperatures, run times, number and composition of primers, detection method for amplification products, amplification efficiency, template composition, complexity of preparation, and commercial availability. Some of the isothermal amplification techniques include strand-displacement amplification (SDA) (Walker et al. 1992), nucleic acid sequence based amplification (NASBA) (Compton 1991, Malek, Sooknanan, and Compton 1994), rolling circle amplification (RCA) (Gusev et al. 2001), nicking enzyme amplification reaction (NEAR) (Van Ness, Van Ness, and Galas 2003), helicase dependent amplification (HAD) (Vincent, Xu, and Kong 2004), loop-mediated isothermal amplification (LAMP) (Notomi et al. 2000), isothermal and chimeric primer-initiated amplification of nucleic acids (ICAN) (Mukai et al. 2007, Uemori et al. 2007), and recombinase polymerase amplification (RPA) (Piepenburg et al. 2006).

Recombinase polymerase amplification (RPA)

RPA is one of the newer isothermal techniques that has many advantages over both PCR and other isothermal amplification strategies. RPA is a low temperature amplification strategy that requires three core enzymes to produce amplification products in a single tube format (Piepenburg et al.

2006). RPA relies on a strand-displacing polymerase, single stranded binding proteins (SSB), and the formation of a recombinase filament complex that is comprised of template specific opposing oligonucleotide primers and a recombinase. The recombinase facilitates the hybridization of the primers to their homologous sequence by scanning the target duplex DNA. SSB assist in the hybridization of the recombinase-primer filaments by binding to displaced strands of DNA and preventing primers from being displaced and re-hybridization of the DNA. Exponential DNA synthesis is initiated by a strand-displacing DNA polymerase without any additional manipulation. Figure 1.4 gives an overview to the RPA reaction (Boyle et al. 2014).

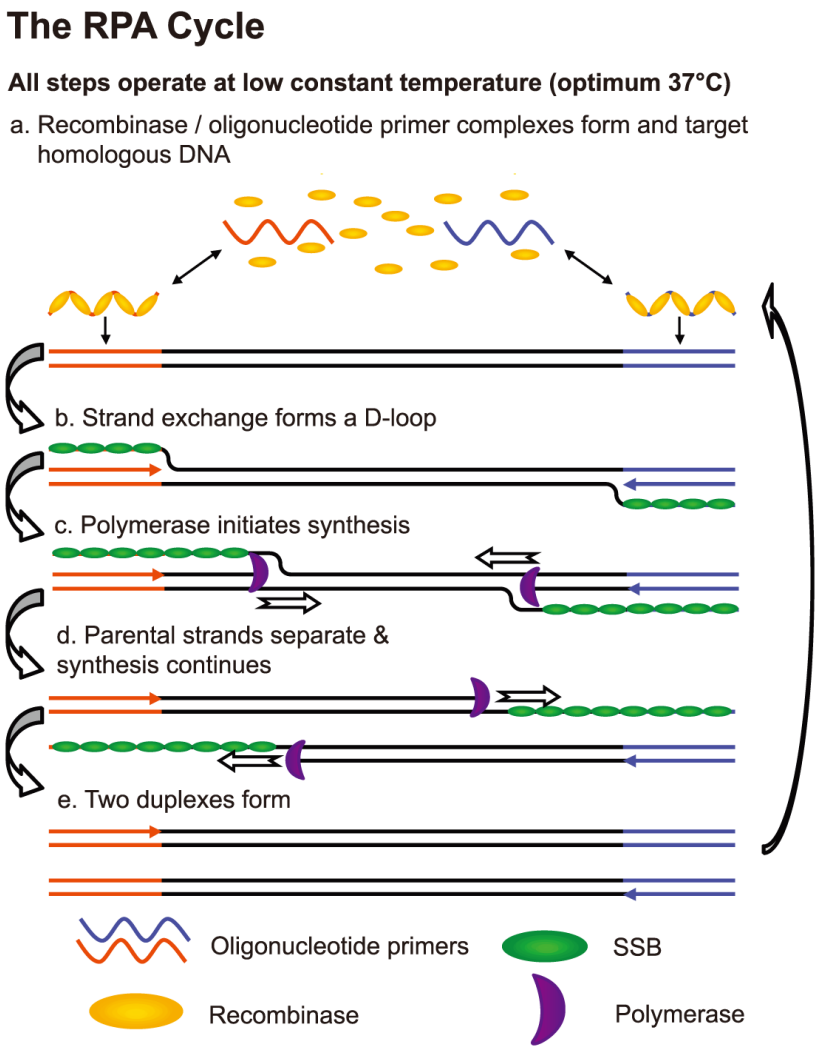


Figure 1.4. Overview of the RPA cycle. From (Boyle et al. 2014).

A number of platforms can be used to monitor or detect amplification products including fluorescent assay, real-time detection by fluorometer, and detection of end products through commercially available lateral flow strips (Deng and Gao 2015). RPA is a DNA amplification strategy, but can be combined with a reverse transcriptase (RT) enzyme for single tube amplification of RNA templates with no additional steps required. RPA kits are commercially

available from TwistDX and allow for simplified amplification by RPA as the kits are supplied as ready to use lyophilized pellets that only need rehydration with reaction buffer containing specific primers and templates of interest. Amplification is initiated by the addition of a magnesium-acetate solution and reactions are typically run for 20 to 30 minutes. Amplicons are usually between 100-200 bp, but longer amplicons up to 1.5 kb have been shown to amplify (Piepenburg et al. 2006). RPA primers are distinct to traditional PCR primers as they are longer for RPA which are typically 30-35 nucleotides. Unlike PCR, melting temperatures are inconsequential, however, primers will perform differently based on sequence. There are no fixed rules that predict how a primer will perform, but the company suggests that long tracks of guanines at the 5' end should be avoided while cytidines/pyrimidines in general encourage the formation of recombinase filaments (TwistDx). GC clamp is also suggested for the 3' end and excessively higher or lower GC content is ill-advised. For detection purposes, modifications to the primer mix are required, as is the formulation of the kit used. TwistAmp basic kits are used for non-fluorescent detection where real-time formats are not required. Reactions that use lateral flow detection would need appropriate primers, dependent on the device purchased. For real-time detection the TwistAmp exo kits are utilized and a TwistAmp exo probe is required. RPA is an attractive alternative for diagnostic purposes as it is easily formatted for the detection technique desired, is fast, simple to use, stable, and capable of both reliable and specific amplification (dependent on primer design).

Scope of Dissertation

The aims of the studies contained within this dissertation are to gain understanding of important viral pathogens and apply knowledge of viral disease to new techniques relevant for viral detection and prevention

strategies. Studies encompassed two crucial areas of viral research: host-pathogen interactions and diagnostics.

Host-pathogen interaction is a broad reaching category that aids in our overall understanding of disease pathogenesis. This knowledge can be utilized for treatment or prevention strategies but also aids in general understanding of parasitic relationships that can be utilized when dealing with emerging diseases that are bound to arise in the future. In the first part of this dissertation, host-viral interactions are examined for two key viral respiratory pathogens: influenza and RSV. The interplay between host genes and viral replication of influenza virus was exploited for the purpose of enhancing the production of influenza virus for vaccine production. In addition, host-viral interactions were examined in mouse embryotic fibroblast cell lines in an attempt to understand the host response to RSV infection.

Diagnostics also play a key role in how we contend with viral disease. The ability to detect viral pathogens affords us the opportunity to provide appropriate care to infected individuals when treatment options are available. It also allows for surveillance of viral disease that is critical for many aspects in our battle to control disease burden. The importance of surveillance is often overlooked, however, viral diagnostics allow for us to track the epidemiological factors, from the spread and distribution of a viral disease, to the incidence and severity of disease in those infected. These items allow us to tract the burden that a viral disease imparts so that we can control the spread of viral disease by quarantine or restricting movement in cases of pandemics or unseasonable epidemics, and grants us the knowledge to focus our resources to those viral diseases with the biggest impact. In the second portion of this dissertation, an isothermal nucleic acid amplification technique was applied to two viral pathogens in need of improved diagnostics.

I believe that this research is innovative and significant, because the new knowledge gained from this study may lead to the development of novel antiviral therapies or prevention strategies with public health significance and the RPA diagnostic tests, following further field-based evaluation, may offer much needed point-of-care diagnosis of disease. I also believe that the findings in this dissertation may lead to the expansion of research with interests from future graduate students, collaborations with other principal investigators and industry.

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Chapter 2. Inhibition of interferon alpha by a peptide-conjugated phosphorodiamidate morpholino oligomer increases influenza virus replication in chicken embryo fibroblast (DF-1) cells; an alternative strategy to increasing vaccine production in cell culture

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Abstract

Influenza is a global health issue causing substantial health and economic burdens in affected populations. Routine, annual vaccination for influenza virus is recommended for all persons older than 6 months of age. Propagation of influenza virus for vaccine production is predominantly through embryonated chicken eggs. Many challenges face propagation of the virus, including, but not limited to, low yields and lengthy production time. The development of a method to increasing vaccine production in eggs or cell lines by suppression of cellular gene expression would be helpful to overcome some of the challenges facing influenza vaccine production. Here we describe the use of a peptide-conjugated phosphorodiamidate morpholino oligomer (PPMO), an antisense molecule, to suppress protein expression of the host gene interferon alpha (IFNα) in chicken embryo fibroblast (DF-1) cells. Suppression of IFNα by PPMO resulted in significantly reduced levels of IFNα protein in treated wells as measured by ELISA and was shown to not have any cytotoxicity to DF-1 cells at the effective concentrations tested. Treatment of the self-directing PPMO increased the ability of the influenza virus to replicate in DF-1 cells. Over a three-fold increase in viral production was observed in PPMO treated wells compared to those of untreated controls, which was observed to be independent of the initial viral input (MOI) studied. The use of PPMO would allow for cell cultures to produce increased levels of influenza for vaccine production or alternatively as a screening tool to cheaply test targets prior to development of permanent knockouts of host gene expression.

Introduction

Influenza viruses belong to the *orthomyxoviridae* family and are responsible for extensive yearly seasonal disease epidemics worldwide (WHO 2014a). Influenza is a clear global public health issue; in the United States alone, it is

estimated that 5%-20% of the population is infected with influenza per year (CDC 2015c). While the young and elderly are more at risk for contracting seasonal influenza, adults are also susceptible to infection. In addition to seasonal disease burden, influenza pandemics are a constant global concern and have the potential to be devastating to human populations based on history of past pandemics (Ng and Gordon 2015, Simonsen et al. 2013, McLaughlin et al. 2015). Vaccination is considered to be the most effective strategy to curtail the annual disease burden of influenza and recommended to those over 6 months of age without contraindications (Grohskopf et al. 2015). The demand and availability for the influenza vaccine has increased rapidly in past years and the CDC projects as many as 156 million available doses for the 2014-2015 season and for the 2015-2016 season, as many as 179 million doses of the influenza vaccine will be available in the United States (CDC 2015a, d).

Influenza virus grown for the seasonal influenza virus vaccine is typically produced in embryonated chicken eggs (Brauer and Chen 2015, Spackman and Killian 2014). Recently in the United States, cell culture-based vaccines have been approved for use, but the egg-based method for producing vaccines still predominates (Buckland 2015). While egg-based methods have been considered superior at producing virus, the production of virus is still limited to a sub-optimal level of ~1 vaccine dose/egg (Durando et al. 2011, Fiore, Bridges, and Cox 2009). Egg-based production technology has several drawbacks most concerning of which is the need for large quantities of embryonated chicken eggs and the long production and lead-up times necessary for this method of virus propagation. Strategies to improve growth of the influenza virus for vaccine production are needed. This is especially true for cell culture methods, which allows for quicker vaccine production for pandemic virus. Improved methods to amplify influenza virus is also critical for

manufacturing of the seasonal vaccine; the demand for which will only continue to grow, ever increasing the strain to resources required for production.

Altering the host cell gene expression in cultured cells to increase the efficiency of viral production is a step to improve vaccine production that is needed. The type I interferon (IFN-α/β) response is a central component in the first line against viral infection (Bonjardim, Ferreira, and Kroon 2009, Haller, Kochs, and Weber 2006, Stetson and Medzhitov 2006, Theofilopoulos et al. 2005, García-Sastre 2001, Wu, Metcalf, and Wu 2011). Since these cytokines play a key role in activation of the host antiviral response, we believe that inhibiting their expression would result in the ability of cultured cells to propagate influenza virus at a much higher rate. Specifically, interferon alpha (IFNα) is the target we have chosen to suppress in an attempt to increase influenza viral replication in cell culture. Traditionally, RNA-interference has been utilized in order to inhibit the expression of targeted genes, including those for influenza (Meliopoulos et al. 2012, Kole, Krainer, and Altman 2012). While their use is widespread, siRNA-based approaches have key undesirable downsides. Intracellular delivery is difficult with siRNAs and their use requires transfection reagents that can be toxic to the cell, and the siRNA themselves have been shown to have nonspecific activity (Saxena, Jónsson, and Dutta 2003, Scacheri et al. 2004, Kole, Krainer, and Altman 2012, Svoboda 2007).

The use of peptide-conjugated morpholino oligomers (PPMO) is an alternative method to regulate expression of host genes without an additional delivery system. PPMOs are a technology that inhibits protein synthesis by the antisense steric-blocking of RNA. PPMOs consist of two covalently linked components. A sequence specific, nucleic acid analog that inhibits gene expression or phosphorodiamidate morpholino oligomer (PMO), and a cell-

penetrating peptide (CPP) that facilities the delivery of the PMO into the cells. PMOs are synthetic molecules modeled after RNA but their structure has two key modifications. Unlike RNA, the bases are bound to six-sided morpholine rings which are connected by a phosphorodiamidate linkage (Summerton and Weller 1997). These modifications make PMOs nuclease resistant and metabolically stable (Hudziak et al. 1996, Youngblood et al. 2007). PMOs have been used for drug development and protection against pathogens (Anantpadma, Stein, and Vrati 2010, Böttcher-Friebertshäuser et al. 2011, Geller 2005, Han et al. 2009, Heald et al. 2015, Lam et al. 2015, Mitev et al. 2009, Tilley et al. 2007, Warren et al. 2015, Yamamoto et al. 2011, Geller et al. 2013, Deas et al. 2005, Stein 2008, Nan et al. 2015), to knockdown of gene expression in a number of systems (Bill et al. 2009, Kos et al. 2003, Mimoto and Christian 2011, Sauka-Spengler and Barembaum 2008, Voiculescu, Papanayotou, and Stern 2008), and a host of other applications due to their solubility, specificity, affordability, and lack of immunogenicity (Luo et al. 2014, Wu et al. 2011, Eisen and Smith 2008, Moulton and Yan 2008). When linked to a CPP, knockdown of gene expression can be accomplished without further manipulation and little toxicity (Moulton et al. 2004, Moulton and Moulton 2010). As an alternative to siRNA knockdown, we examined the use of PPMO to inhibit the expression of the host gene IFNα to increase influenza virus production in DF-1 chicken embryo fibroblast cells.

Materials and Methods

Cells and Virus

Chicken embryo fibroblast cell (DF-1), Madin-Darby canine kidney (MDCK) cell, and Vero cell cultures were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in 5% CO_2.

Influenza virus used in this study was A/PR/8/34 (H1N1) obtained from American Type Culture Collection (ATCC, Manassas, VA). Virus was grown in embryonated chicken eggs following standard propagation techniques and stored in aliquots at -80°C then titered by viral plaque assay in MDCK cells (Balish, Katz, and Klimov 2013, Brauer and Chen 2015).

Plaque assay

A monolayer of MDCK cells was grown to 90% confluency, then infected for 1 hour at 37°C with diluted viral sample. Virus samples were serially diluted to appropriate levels in cell culture medium. Infectious media was removed, cells were washed with PBS, and plaque overlay media applied to wells. Overlay media contained 1% low melting temperature agarose and 1.2 µg/ml L-1-tosylamide-2-phenylmethyl chloromethyl ketone (TPCK)-treated trypsin in Eagle's minimal essential medium (EMEM) supplemented with glutamine, antibiotics, and 7.5% BSA. Once the overlay media was solidified, plates were inverted and incubated for five days followed by fixation with 4% paraformaldehyde for 2 hours. Once fixed, paraformaldehyde and plaques were removed, and cells stained with 0.5% crystal violet solution for 30 minutes. Crystal violet was removed, plates gently rinsed with water, allowed to dry, then plaques were counted. Titer of samples determined by plaque assay was expressed as PFU/ml.

PPMO

A PMO against IFNα was designed based on the NCBI Reference Sequence NM_205427.1. A nonsense PPMO was also designed and the PPMOs ordered from Gene Tools LLC (Philomath, OR, USA). The PPMO was covalently attached to the CPP (RXR) 4x as described previously (Abes et al. 2006). Lyophilized PPMO was resuspended with sterile distilled water to a stock solution of 1mM and stored at 4°C. Table 2.1 summarizes the sequence and nomenclature for the CPP and PMOs used.

Replication in multiple cell lines

The replication of Influenza virus in MDCK, DF-1, and Vero cells was determined by titration of virus from cell culture supernatants. Plaque assay on MDCK cells was utilized to determine the viral titer produced for each cell type with three replicates and values reported as PFU/ml. Briefly, each cell type was infected with a multiplicity of infection (MOI) of 0.01, 0.1, 0.5, and 1.0 for 1 hour at 37°C. Following absorption, infectious media was replaced with fresh culture medium. Cell supernatants were collected 24 hours post-infection (hpi) and plaque assay was performed.

Statistical analysis

One-way ANOVA was performed using GraphPad Prism version 6.01 (GraphPad Software, San Diego,CA, USA). When necessary, multiple comparisons were analyzed by Tukey *post hoc* test.

ELISA

ELISA was used to assess the change in IFNα protein expression attributed to treatment with the PPMO. ELISAs were performed according to the abcam indirect ELISA protocol. Following these guidelines, Nunc-Immuno MicroWell 96-well plates (Nunc, Rochester, NY, USA) were coated with 50 µl of sample antigen and incubated at room temperature (RT) for 2 hours. Samples consisted of cell supernatants undiluted or diluted two-fold with PBS in triplicate wells. After coating with sample, wells were washed three times with 200 µl PBS and blocked with 200 µl of a 5% non-fat dry milk blocking solution for 2 hours at RT followed by two additional washes. Wells were then coated with 100 µl of primary antibody, mouse monoclonal anti-chicken interferon alpha MCA2412 (abD Serotec, Atlanta, GA, USA), covered and incubated at 4°C overnight. Wells were washed four times, then 100 µl of conjugated secondary antibody was added for 2 hours at RT, followed by an additional four washes. Detection was achieved by using TMB (3,3’,5,5’-

tetramethylbenzidine) Conductivity One Component HRP Microwell Substrate (SurModics Inc., Eden Prairie, MN, USA). 100 µl of TMB was added to wells and incubated for 15-30 min. BioFX 450nm Liquid Stop Solution (SurModics Inc.) was used to stop the reaction and plates were read at an optical density of 450 nm on an Ultramark Microplate Reader (Bio-Rad, San Diego, CA, USA).

Cytotoxicity assay

Cell viability of PPMO treated DF-1 cells was assessed by the MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; thiazolyl blue) colorimetric assay (Sigma- Aldrich, St. Louis, MO, USA). 100 µl of working stock MTT (10 µl MTT stock solution (5mg/mL), 90 µl media) was added to each sample well of a 96-well plate. The plate was incubated at 37°C for 1.5 hours or until color developed. MTT reagent was removed from cells and 100 µl DMSO added to solubilize the formazan crystals. DMSO in blank wells were used as control. Plate was mixed gently to homogenize the solution and absorbance measured at 550nm on the Ultramark Microplate Reader (Bio-Rad). The MTT assay was performed in triplicate wells, 24 hours post treatment with PPMO or control CPP, to obtain the values plotted.

Influenza viral replication following treatment of PPMO

DF-1 cells were treated with CPP, anti-IFNα PPMO or nonsense PPMO diluted in culture media at concentrations described in the results two hours prior to infection at MOI 0.1 unless otherwise indicated. After removing culture media, infectious media containing PPMO or control peptide was added and allowed to infect for 1 hour. Infectious media was then replaced with fresh culture media again containing PPMO or CPP at appropriate concentrations. Cells were incubated for 24 hours then culture supernatants were harvested and influenza titrated. Replication of Influenza virus was measured by plaque assay on MDCK cells following standard methods.

Results

Comparison of influenza virus replication in three cell lines

Replication of Influenza virus, measured by plaque assay, was compared in three cell lines. Influenza replication at 24 hpi for MOIs of 1.0, 0.5, 0.1, and 0.01 was observed for each of the cell lines: MDCK, Vero, and DF-1. Replication was observed in MDCK and Vero cells as they are the classic cell lines used for influenza propagation and vaccine production. In addition, DF-1 cells were also surveyed, as they are compatible with the PPMO designed to target chicken IFNα used in this study. For each MOI examined, influenza replication in each cell type was represented as the log of each titer calculation as seen in Figure 2.1. Virus propagation in DF-1 cells is comparable to the yields observed for both Vero and MDCK. While the titer observed in DF-1 cells did not exceed that of MDCK, it did outperform Vero cells for replication of the virus.

Viral replication in PPMO treated DF-1 cells

The effect to influenza virus replication by treatment of DF-1 cells with the PPMO targeting chicken IFNα was examined. The viral replication of influenza was measured by plaque assay 24 hpi with an MOI of 0.1 in PPMO treated DF-1 cells. Significant increase in viral production was observed in each experiment for two of the PPMO conditions tested (Figure 2.2). PPMO concentrations of 10 µM in DF-1 cells was sufficient to significantly increase influenza replication (column E; $p < 0.01$, ANOVA) using the standard protocol for introduction of PPMO to the cells (2 hours prior to infection). When PPMO was added to cells earlier (18 hours prior to infection) than the standard protocol, an even greater increase to influenza replication was observed, column F of Figure 2.2 ($p < 0.001$, ANOVA). An over 2-fold increase in viral replication was observed for DF-1 cells treated with 10 µM PPMO for the standard 2 hour pre-treatment condition and an over 3-fold

increase in replication for the same concentration of PPMO added 18 hours before infection. A lower concentration of PPMO was also tested, but did not result in a significant increase over all controls. In addition to the baseline replication of the untreated control, replication in wells treated with PPMO controls was also observed. Neither the CPP control nor the nonsense PPMO control produced influenza significantly different from untreated controls, indicating our PPMO targeting IFNα was responsible for the increased viral replication seen in non-control wells.

To further investigate the increase observed in influenza replication due to treatment of anti-IFNα PPMO, viral titers were compared between untreated control samples and 10 μM anti-IFNα PPMO treated samples at three MOIs. DF-1 cells were treated with PPMO 18 hours prior to infection as earlier pre-treatment resulted in higher titers (Figure 2.2) in prior experiments. Infection of influenza virus was performed at MOIs of 0.1, 0.5 and 1.0 and cell supernatants were collected for titration by plaque assay at 24 hpi. Similar to the previous experiments, treatment with anti-IFNα PPMO resulted in increased viral titers compared to untreated controls for each of the MOIs examined (Figure 2.3). Results from the 0.1 MOI further corroborated prior replicated experiments where viral replication in DF-1 cells increased greater than 3-fold from the control and an approximately 0.5 log increase. A similar increase was also observed for 0.5 MOI and 1.0 MOI over similarly infected, untreated controls.

PPMO treatment alters the expression of the IFNα protein

Based on our experiments examining the effect of PPMO in DF-1 cells, the addition of anti-IFNα PPMO increases viral replication in the DF-1 cell line. To confirm that expression of IFNα protein is being modulated by the addition of the anti-IFNα PPMO, protein levels were compared for untreated DF-1 control cells and those treated with the previously established effective dose of 10

μM for both a control nonsense PPMO and anti-IFNα PPMO by ELISA. Assay controls were also included to account for background absorbance. The absorbance measured for the treatment control wells (untreated DF-1 cells and nonsense PPMO treated DF-1 cells) was not significantly different. Control samples compared to those of anti-IFNα PPMO treated cells displayed significantly ($p < 0.01$, ANOVA) higher absorbance indicating reduced levels of IFNα protein in anti-IFNα PPMO treated wells (Figure 2.4). This result verifies that treatment of DF-1 cells with anti-IFNα PPMO results in a measurable and significant reduction in the amount of chicken IFNα protein produced in these cells. This knockdown inhibits the production of IFNα protein and allows for increased replication of the influenza virus.

Cytotoxicity assay

To ascertain cytotoxicity associated with treatment of DF-1 cells with the PPMO, MTT was used to test cell viability. Figure 2.5 depicts the cytotoxicity of the PPMO or control treatments in DF-1 cells; results are plotted as absorbance from performing the MTT assay. Controls are plotted as A-C and represent averaged reading for viability in **A** control untreated DF-1 cells, **B** control nonsense PPMO (10 μM), and **C** control CPP (10 μM). The viability of cells treated with anti-IFNα PPMO are plotted as D-F and represent averaged readings from **D** anti-IFNα PPMO (5 μM), **E** anti-IFNα PPMO (10 μM), and **F** anti-IFNα PPMO (10 μM) 18 hour pre-treatment. All values have been normalized to the DMSO control for the MTT assay. Based on the MTT assay absorbance readings, none of the PPMO treatments resulted in cytotoxicity significant from the control, untreated wells. This includes the anti-IFNα PPMO treated wells in addition to the PPMO control wells containing nonsense PPMO and CPP. The addition of PPMO to DF-1 cells did not result in any visible changes to cell viability including morphology or cell density

variations. Overall, treatment of the anti-IFNα PPMO and control compounds at the concentrations tested in this study were not cytotoxic to DF-1 cells.

Discussion

The global impact of influenza disease marks the influenza virus a major health concern. While influenza vaccination is available, several limitations in production methods highlight the need for improved and/or alternative methods for vaccine production. Here we describe the use of PPMO technology targeting the chicken host gene IFNα to reduce this anti-viral protein production, thereby increasing the ability of influenza to replicate in DF-1 cells. We show that in the DF-1 cell line, influenza A/PR/8/34 replicates to titers midway between MDCK and Vero cells, both of which are currently used in the manufacture of influenza vaccine. The addition of IFNα targeting PPMO to cells at 10 μM displayed no toxicity and induced increased growth of influenza virus compared to controls. We demonstrated that increased virus production was verified at three different MOIs ranging from 0.1 to 1.0 indicating that the effect of treatment with the anti-IFNα PPMO was independent of viral input. In addition, we confirm that the anti-IFNα PPMO resulted in a significant reduction in IFNα protein produced in DF-1 cells compared to untreated and nonsense PPMO controls.

While the manufacture of influenza vaccines predominantly utilizes embryonated eggs for influenza virus production, cell culture-based vaccines are approved for use and gaining traction. Cultured virus is frequently grown in MDCK and Vero cells, but other cells lines are being investigated and submitted for approval for use in vaccine production. We show that DF-1 cells can grow influenza at baseline levels comparable to these already established and approved cell lines. Here we describe an alternative method to enhance vaccine production by the addition of PPMO to target IFNα,

increasing viral production in these cells. In addition to directly modulating chicken cell lines to increase production of influenza for vaccine production, the use of PPMO could be a cheap and attractive method of screening targets for more permanent host gene modification. Cells with permanent knockouts would be ideal but costly and time consuming to produce without cheaper and quick screening of intended targets of host gene modifications.

Chicken cells were used in this study in an effort to modulate gene expression in both cell culture as well as embryonated chicken eggs. While a small experiment was conducted using the anti-IFNα PPMO (data not shown) in embryonated eggs, knockdown of protein and increased viral production was only established in cell culture. There is some question as to the theoretical success of modulation of anti-viral genes in the egg production system as there is limited insight to the ontogeny of the chicken immune system. Based on methodologies for influenza growth in embryonated chicken eggs, and data available for the induction of IFNα response in chicken embryos, anti-viral genes may prove to be unusable as targets in the egg (Karpala et al. 2012, Peters et al. 2003). While alternative targets for PPMO based enhanced production of influenza virus in eggs would be a valuable goal, it is beyond the scope of this study.

Cell based production of influenza has several advantages as it permits all types of influenza virus propagation, reduces lead-up time, maintains more aseptic conditions, produces purer viral cultures, and eliminates several egg related issues including allergy and flock control issues associated with egg-based manufacture of the influenza virus. The use of PPMO to increase viral titer in culture is a much needed step in improving the influenza vaccine, especially considering the allergy risk of egg vaccines. Children are one of the hardest hit groups for seasonal influenza. Globally, influenza is estimated to infect 30% of children each year (WHO 2014a) and egg allergy is the second

most frequent food allergy among children (Eggesbø et al. 2001). While a recent study indicated severe reactions are rare for those with egg allergy (Des Roches et al. 2012), adverse reactions do occur due to egg propagation and eliminating this altogether would be ideal.

Here we show a method to improve influenza virus production in cell culture by targeting the host anti-viral interferon response. Our self-directed PPMO targeting IFNα increases viral production in DF-1 cells by knockdown of protein expression in an easy and nontoxic manner. These results are promising in the move to improve influenza vaccine production. Future work could address additional targets and/or multiplexing PPMO targets to maximize the replication of influenza virus and improve even further the amplification of virus.

Acknowledgements

We would like to thank Gene Tools LLC (Philomath, OR, USA) for providing PPMO. We would also like to thank Dr. Ralph A. Tripp and Ms. Jackelyn Crabtree from Dr. Tripp's lab for providing DF-1 cell line. This research was funded by Agricultural Experimental Station and Oregon State University College of Veterinary Medicine.

Table 2.1. Sequences and nomenclature for the cell penetrating peptide (CPP) and phosphorodiamidate morpholino oligomer (PMO)s used in this study.

Component	Name	Sequence 5'-3'
CPP[a]	(RXR)4X	(RAhxR)4AhxB[b]
PMO	Nonsense PMO	CCTCTTACCTCAGTTACAATTTATA
PMO	IFNα PMO	ACTCGCGTTGTGCTGGGCA

[a] CPP attached to 5' end of PMO to make PPMO
[b] Ahx = 6-Aminohexanoic acid. B = β-Alanine.

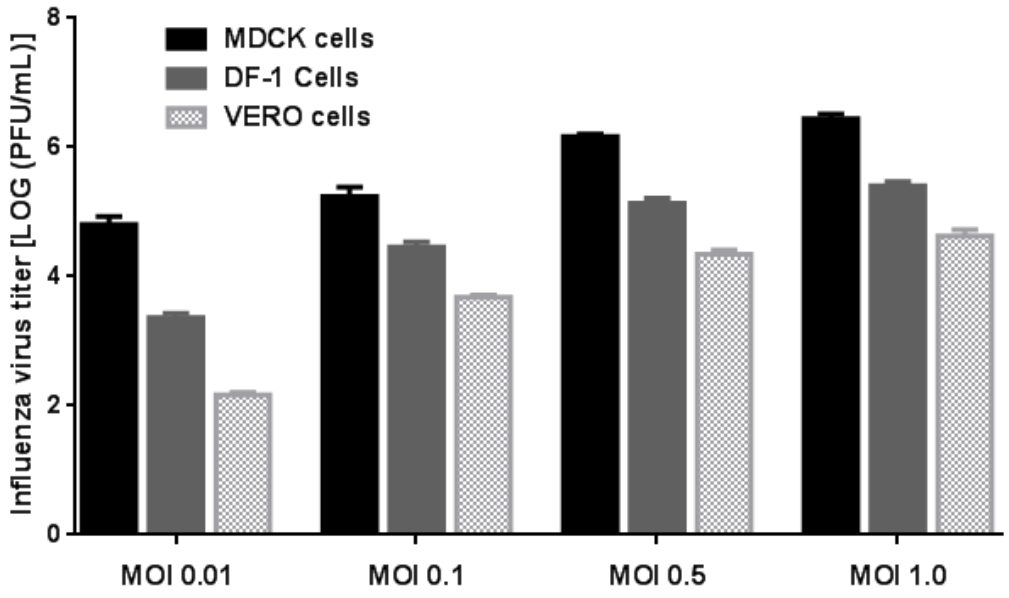

Figure 2.1. Comparison of influenza virus replication in three cell lines: MDCK, DF-1, and Vero. Each cell type was infected at four MOIs and virus replication was measured at 24 hpi as virus titer (log PFU/ml) by plaque assay. Each bar represents data from triplicate wells and error bars indicate standard deviation (SD).

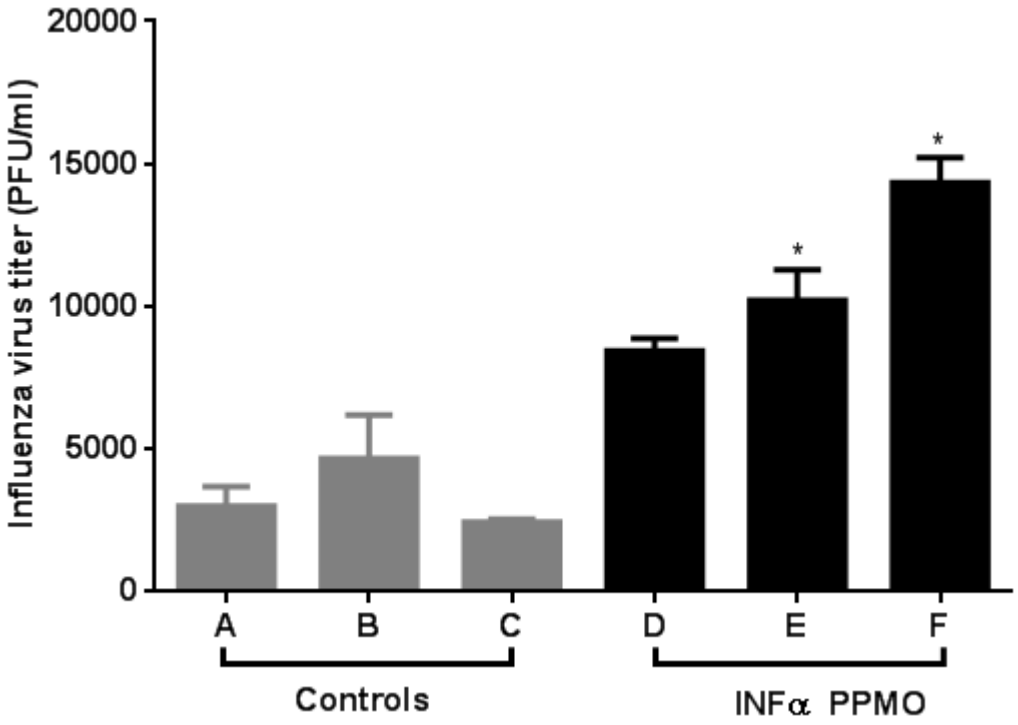

Figure 2.2. A representative graph of influenza virus replication after treatment with PPMO targeting IFNα and infection with influenza at MOI 0.1 in DF-1 cells. Virus was detected from cell supernatants and titered as PFU/ml by plaque assay. Control groups A-C represents: **A** control untreated DF-1 cells; **B** control nonsense PPMO (10 µM); **C** control CPP (10 µM). Wells treated with IFNα specific PPMO represent groups D-F: **D** anti-IFNα PPMO (5 µM); **E** anti-IFNα PPMO (10 µM); **F** anti-IFNα PPMO (10 µM) 18-hour pre-treatment. Error bars represent standard deviation (SD) of means calculated from triplicate wells. Asterisks indicate significant differences ($p < 0.01$) from controls.

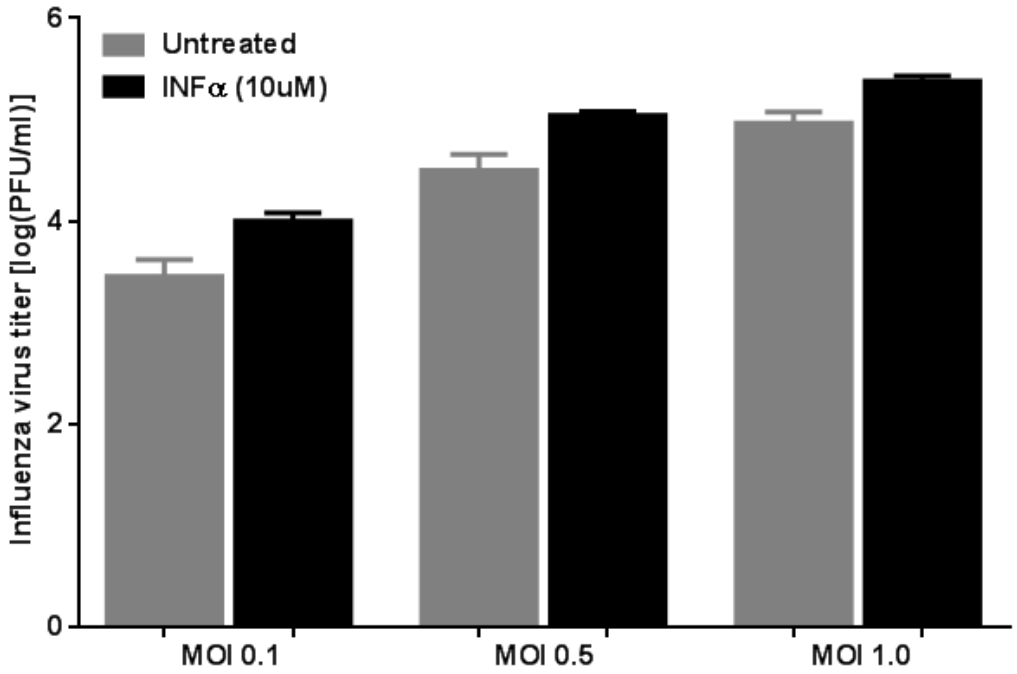

Figure 2.3. Comparison of influenza replication between untreated and IFNα specific PPMO treated DF-1 cells. DF-1 cells were untreated or treated with 10 µM anti-IFNα PPMO then infected at various MOIs. Cell supernatants were collected 24 hpi and virus titered by plaque assay. Error bars represent standard deviation (SD) of means calculated from triplicate wells.

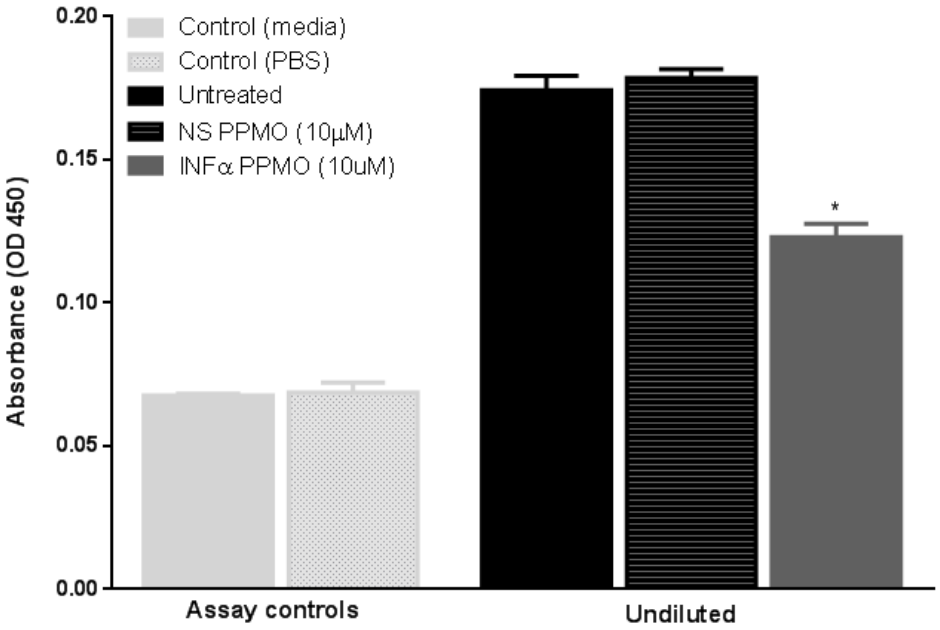

Figure 2.4. Difference in IFNα protein levels between DF-1 cell sample control wells (untreated and nonsense PPMO treated) and cells treated with PPMO targeting IFNα measured as absorbance by ELISA according to the abcam indirect ELISA protocol. Assay controls for background absorbance for both the diluent (Control (PBS)) and the sample media (Control (media)) were included and serve as a baseline for absorbance readings. Absorbance is plotted for the undiluted DF-1 cell supernatants of the untreated control, nonsense (NS PPMO (10 µM)) PPMO control, and anti-IFNα PPMO (10 µM) treated samples. Error bars represent standard deviation (SD) of means calculated from triplicate wells and significant differences are indicated by an asterisk ($p<0.01$, ANOVA, Tukey post hoc).

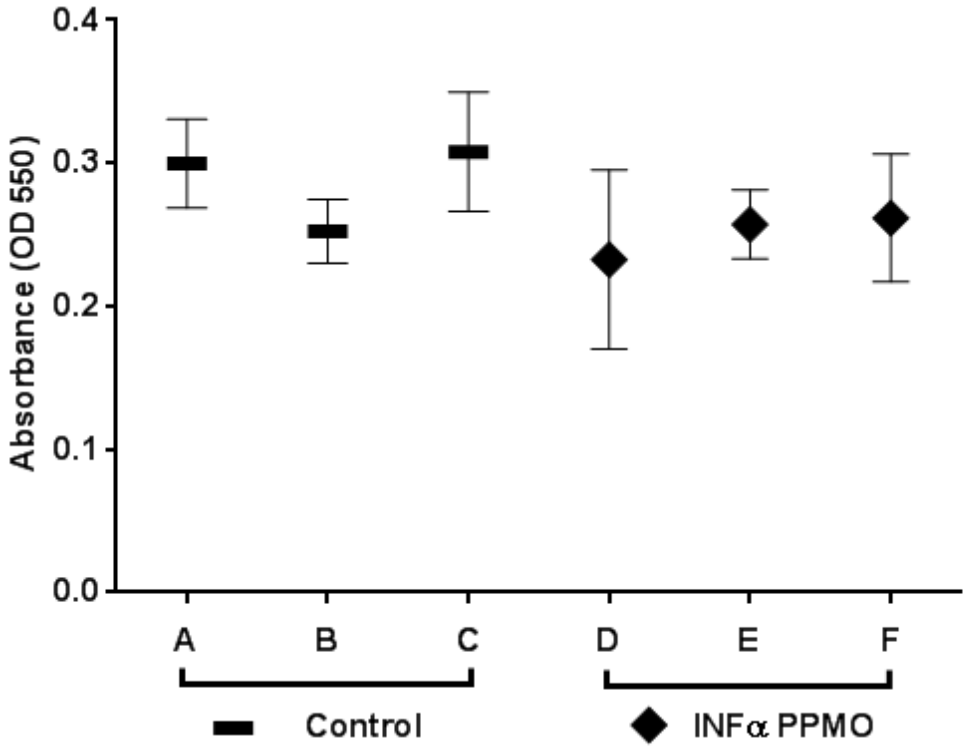

Figure 2.5. Cytotoxicity of PPMO targeting IFNα in DF-1 cells plotted as absorbance and measured by the MTT assay for cell viability. The MTT assay was performed in triplicate, 24 hours post treatment with PPMO or control CPP, to obtain the values plotted. **A** control untreated DF-1 cells; **B** control nonsense PPMO (10 µM); **C** control CPP (10 µM); **D** anti-IFNα PPMO (5 µM); **E** anti-IFNα PPMO (10 µM); **F** anti-IFNα PPMO (10 µM) 18 hour pre-treatment.

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Chapter 3. Myeloid Cell Leukemia -1 knockout leads to increased viral propagation of Respiratory Syncytial Virus in mouse embryonic fibroblast cells

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Abstract

Respiratory Syncytial Virus (RSV) is a leading cause of lower respiratory tract infection in children and elderly worldwide; there is no vaccine or effective therapy available. Myeloid Cell Leukemia -1 (Mcl-1) has been shown to be upregulated in RSV infection. In this study we examine the role Mcl-1 plays in RSV infection and aim to determine the significance of Mcl-1 upregulation in infection. We observed RSV replication in mouse embryonic fibroblast (MEF) cells and demonstrate that MEF cells lacking Mcl-1 are more permissive to RSV infection, leading to significantly increased production of the virus. MEF cells lacking Mcl-1 exhibited increases in viral titers, syncytial formation, and late stage apoptosis. Our work provides evidence that Mcl-1 upregulation in RSV infection would not be beneficial to the virus, rather Mcl-1 upregulation is most likely an antiviral strategy and suggests a possible function for Mcl-1 external to apoptosis regulation.

Introduction

Acute respiratory infections (ARI) are a significant contributor to the global health burden. Human Respiratory Syncytial Virus (RSV) is a leading cause of respiratory infections and associated illnesses contribute significantly to morbidity and mortality at all ages; although RSV predominantly causes severe disease in children, elderly, and those with compromised immune systems (Branche and Falsey 2015, Falsey 2007, Langley and Anderson 2011, Murata and Falsey 2007, Nair et al. 2010, Walsh and Falsey 2012, Verani et al. 2013, Lee et al. 2013). The annual incidence of RSV infections in children under the age of five is estimated at nearly 40 million cases and by two years of age nearly all children in the USA have been infected by the virus at least once (Nair et al. 2010, Ogra 2004, Borchers et al. 2013).

RSV is an enveloped virus of the *Paramyxoviridae* family with a single negative sense RNA genome ~15,000 bp comprising 10 genes encoding 11 proteins (Borchers et al. 2013). RSV nonstructural (NS) proteins NS1 and NS2, and envelope fusion and small hydrophobic (SH) proteins have each been shown to play a role in apoptotic events associated with RSV infection (Li et al. 2015, Eckardt-Michel et al. 2008, Bitko et al. 2007).

Apoptosis is a critical factor in host-viral interactions and many viruses either induce or suppress cell death during infection to enhance fitness and support viral propagation (McLean et al. 2008). Evasion of apoptosis is a common characteristic of viral infection and many viruses encode anti-apoptotic virulence factors that inhibit apoptosis, while others modulate apoptotic pathways to trigger apoptosis leading to increased propagation and/or a more pathogenic virus (Liang, Oh, and Jung 2015, Ghosh Roy et al. 2014, Krejbich-Trotot et al. 2011). An example of the inconsistent viral manipulation of apoptosis is shown by the tumor suppressor protein p53. This protein is an important positive regulator of apoptosis that is targeted for destruction by adenovirus and papillomavirus and inhibited in Kaposi's sarcoma-associated herpesvirus, but has been shown to be positively modulated in Rift Valley fever, HIV, influenza, and West Nile viral infection to induce apoptosis (Austin et al. 2012, Chudasama et al. 2015, Huibregtse, Scheffner, and Howley 1991, Steegenga et al. 1998, Suo et al. 2015, Verma et al. 2011, Wang et al. 2014, Yang et al. 2008, Eckardt-Michel et al. 2008).

RSV modulation of apoptosis has been investigated and various mechanisms are exploited in the regulation of apoptosis following infection. Previous studies have revealed the complex nature of apoptosis modulation in RSV infection and data is often conflicting between the studies. It seems that early apoptosis is curbed through various mechanisms including the degradation of p53, delay by activation of the epidermal growth factor receptor and

suppression by the upregulation of anti-apoptotic host factors like NF-KB (Groskreutz et al. 2007, Bitko et al. 2007, Thomas et al. 2002, Lindemans et al. 2006, Monick et al. 2005). However, during later stages of infection, apoptosis induction has been demonstrated to be mediated by TRAIL, FasR, ER stress-activated caspase-12, iNOS/NO, and through p53 regulated apoptosis triggered by the RSV fusion protein (Bem et al. 2010, Bitko and Barik 2001, Mgbemena et al. 2013, Eckardt-Michel et al. 2008, Kotelkin et al. 2003, O'donnell, Milligan, and Stark 1999). The modulation of apoptosis is an intricate balance of inducement or suppression at crucial time stages in RSV infection that is not completely understood.

Myeloid Cell Leukemia -1 (Mcl-1) is an anti-apoptotic regulator of apoptosis, shown to be upregulated in RSV infection as early as 2 hours after initial infection (Figure 3.1) (Lindemans et al. 2006, Lupfer 2009). There are over 12 core BCL-2 family proteins that function in the regulation of apoptosis; pro-apoptotic effectors (BAX, BAK, BOK, BID, BIM, BAD, NOXA, PUMA) and antagonizing, anti-apoptotic members (BCL-2, BCL-W, BCL-XL, Al, and Mcl-1) (Youle and Strasser 2008). Mcl-1 has several functions dependent on its localization in the cell, but it performs its apoptotic role when localized to distinct mitochondrial subcompartments on the outer mitochondrial membrane (OMM) where it antagonizes BAX and BAK (Perciavalle et al. 2012). The role of Mcl-1 in apoptotic modulation during RSV infection has yet to be defined as pro or anti-viral in nature. Here, we demonstrate that mouse embryonic fibroblast (MEF) cells lacking Mcl-1 propagate RSV virus much better than unmodified wild type (WT) MEF cells. We find distinct morphological changes in Mcl-1 knockout (∆Mcl-1) MEF cells infected with RSV, and examine apoptotic modulation effects that may indicate a functionality to Mcl-1 separate from its classical role in apoptosis regulation during RSV infection.

Materials and Methods

Cells and virus

MEF cells were kindly provided by Dr. Joseph T. Opferman (St. Jude Children's Research Hospital, Memphis, TN, USA) and the generation of the ΔMcl-1 MEF mutation is previously described (Opferman et al. 2003). Murine RAW 264.7 macrophage cells were kindly provided by the Bermudez Laboratory (Oregon State University, Corvallis, OR, USA). All other cells used for plaque assays, viral propagation, or for other experimental purposes were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured and maintained in cell culture medium consisting of Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in 5% CO_2.

RSV VR-1540 HRSV-A2 (ATCC) was used for all experiments involving RSV. Viral stocks were prepared following the general guidelines previously published with modifications (Ueba 1978). Briefly, HEp-2 cells were infected with RSV at an MOI of 0.5 and virus was propagated for two days; a single plaque was harvested and used to infect a new T75 flask of HEp-2 cells. Following a subsequent three day incubation, the cells and media were collected, vortexed, and cells pelleted by centrifugation for 10 minutes at 3,000 x g at room temperature (RT). Resulting supernatant was overlaid onto a 30% sucrose gradient (30% sucrose w/v in 1M MgSO4, 50mM HEPES, 150mM NaCl) and centrifuged at ~60,000 x g for 30 min at 4°C. Pelleted virus was collected and centrifuged once more to concentrate and purify the virus further. Viral aliquots were snap frozen in a dry ice ethanol bath then stored at -80°C until used. Titration of stock virus was determined by plaque assay at regular intervals.

For influenza replication in MEF cells, influenza A/PR/8/34 (H1N1) virus was used. Stock virus was grown in embryonated chicken eggs following standard

propagation techniques and stored in aliquots at -80°C until titration by plaque assay (Brauer and Chen 2015, Balish, Katz, and Klimov 2013).

Plaque assay for RSV

Viral titers were determined by plaque assay using > 90% confluent HeLa cells. Samples were serially diluted to appropriate levels in culture medium, then added to triplicate wells and allowed to infect for one hour at 37°C. Infectious media was removed by aspiration and cell monolayers covered with overlay medium (1% low melting point agarose in DMEM supplemented with 10% FBS). Once overlay was solidified into plugs, plates were inverted and incubated at 37°C for four days. Cells were then fixed with 4% paraformaldehyde for two hours, and agarose plugs removed. Plaques were visualized by immunostaining. Quickly, wells were blocked with 5% dry milk blocking in PBS then probed using the primary goat polyclonal anti-RSV antibody (AB1128 Millipore, Billerica, MA, USA). Following three washes with blot wash buffer (0.05% Tween 20 in 1X PBS), wells were incubated with HRP-conjugated donkey anti-goat secondary antibody (Rockland, Pottstown, PA, USA), washed three additional times then visualized by the chromogenic substrate TMB (3,3',5,5'-tetramethylbenzidine) (BioFX, Owings Mills, MD, USA). Plaques were counted and expressed as the standard plaque forming units (PFU)/ml.

Plaque assay for influenza virus

A 90% confluent monolayer of MDCK cells was infected for one hour at 37°C with diluted viral sample. Virus samples were serially diluted to appropriate levels in cell culture medium. Following Infection, media was removed, cells were washed with PBS, and overlaid with Eagle's minimal essential medium (EMEM) supplemented with glutamine, antibiotics and 7.5% bovine serum albumin (BSA), containing 1% low melting temperature agarose and 1.2 µg/ml TPCK-treated trypsin. Overlay medium was allowed to solidify, then plates

were inverted and incubated at 37°C for five days. Monolayers were fixed with paraformaldehyde for two hours, plugs removed and cells stained with 0.5% crystal violet solution for 30 minutes. Once the stain was removed, wells were rinsed, dried and counted to obtain PFU/ml values.

Replication experiments

The replication of RSV and influenza virus in the MEF cell lines was assessed and compared. In all cases, MEFs were seeded one day prior to replication experiments. Cells were infected with the appropriate virus at the MOIs indicated for each experiment and virus was allowed to replicate for the time specified. Cell supernatants were collected to assess replication by viral titration using the applicable plaque assay methodology.

Apoptosis Assay

A day prior to conducting the assay, a clear bottom 96-well plate (Perkin Elmer, Waltham, MA, USA) was seeded with either WT type or ΔMcl-1 MEFs. Cells were infected with RSV at an MOI of 2 or mock infected with fresh culture medium. Triplicate wells for both MEF cell type and infection type were assayed for caspase activity every 12 hours for a total of 48 hours post infection (hpi). Caspase activity was measured by the Apo-ONE Homogeneous Caspase-3/7 Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. Cleavage of the profluorescent caspase substrate (supplied by the assay) via caspase 3/7 results in the fluorescent rhodamine 110 compound that can be measured by a fluorescent plate reader. Fluorescent readings of caspase activity were measured at 485 nm/535 nm (excitation/emission) using the Tecan F200 plate reader (Tecan Group Ltd, Männedorf, Switzerland).

Confocal microscopy

MEF cells were grown on sterile, round glass coverslips coated with poly-L-lysine then infected with RSV at an MOI of 2 for one hour. Following infection,

infectious media was replaced with fresh culture media. At appropriate time points (12, 24, 48, and 72 hpi), cover slips were washed with cold PBS then fixed with 4% paraformaldehyde. Each population of cells was then permeabilized with 0.2% Triton X-100 in PBS then washed again in PBS. Cells were blocked with 2% BSA solution in PBS, stained with primary goat polyclonal anti-RSV (EMD Millipore, Billerica, MA, USA) antibody diluted 1:100, then washed with blot wash buffer. Cells were then stained with AlexaFluor 488 rabbit anti-goat secondary antibody (Life Technologies, Carlsbad, CA, USA) at a 1:200 dilution. Following staining, coverslips were mounted onto glass slides using Vectasheild mounting media with DAPI nuclear stain (Vector Labs, Burlingame, CA, USA). Immunofluorescently stained cells were viewed and images acquired via confocal microscopy with a Leica Zeiss LSM510 META with Axiovert 200 motorized microscope and LSM software. Control cells were also viewed to control for aberrant probing of antibodies. An infected control was stained with only secondary and an uninfected control was stained as described above for infected MEFs. The 63X oil objective was used for all visualization and subsequent capturing of representative micrographs. A minimum of 30 fields of view were observed per slide for each of the MEF cell types and a minimum of six representative images were acquired per cell type per time point examined through the LSM software.

Western blots

Protein was collected from WT and ΔMcl-1 MEF cell cultures along with cultures serving as control cell lysate samples of HeLa and RAW cells. Protein was extracted using RIPA buffer (Thermo Scientific, Wilmington, DE, USA) with 1X ProteaseArrest (G Biosciences, Maryland Heights, MO, USA) to lyse cell cultures following manufacturer’s instructions. Cell lysates were prepared for SDS-PAGE electrophoresis by mixing NuPAGE LDS sample

Buffer (Life Technologies), 500 µM reducing agent (DTT), cell lysate, and DI water to a final 10 µl volume. Samples were heated at 70°C for 10 minutes then loaded onto a NuPAGE Bis-Tris Precast Gel (Life Technologies) with Prestained Broad Range SDS-PAGE Standards (BioRad, Hercules, CA, USA) in the XCell *SureLock* Mini-Cell system (Invitrogen, Carlsbad, CA, USA) with MES SDS running buffer. Samples were electrophoresed for 40 minutes at 200 V, then transferred to a nitrocellulose membrane by the semi-wet transfer unit, XCell II Blot Module (Invitrogen) for 90 minutes at 30 V. Membranes were then blocked overnight at 4°C with Near Infra-Red Blocking Buffer (Rockland, Pottstown, PA, USA). Blots were probed with a primary mouse β-Actin antibody (Santa Cruz Biotechnology, Dallas, TX, USA) as a loading control, washed three times with blot wash then probed with a second primary antibody for the target Mcl-1 via rabbit Mcl-1 (D35A5) antibody (Cell Signaling, Danvers, MA, USA). Blots were washed again then incubated with fluorescent secondary antibodies IRDye800 conjugated anti-rabbit IgG and IRDye700 conjugated anti-mouse antibodies (Rockland). Labeled bands were analyzed on the Odyssey infrared imaging system (Li-COR Biosciences, Lincoln, NE, USA)

Apoptosis induction in WT MEF by camptothecin

WT MEFs that were seeded into triplicate culture wells for each treatment group measured (camptothecin (CPT) treated and DMSO control wells) were infected with RSV at an MOI of 2. Following a one hour incubation at 37°C, infectious media was aspirated and replaced with fresh culture media containing either CPT to induce apoptosis or DMSO to control for the DMSO incorporated due to treatment with CPT. The percentage of DMSO was equal to that found in the respective CPT treated wells for the total volume added to each well. One set of cells received either 2 µM CPT or 0.1% DMSO while another set was treated with 4 µM CPT or DMSO at 0.2% of the total culture

media volume. Virus was allowed to replicate for 24 hours before cell culture supernatants were collected and viral titers for each group were assessed by plaque assay.

Apoptosis inhibition in ΔMcl-1 MEF by Z-VAD-FMK

ΔMcl-1 MEF cells were seeded into triplicate culture wells for each treatment group; Z-VAD-FMK (R&D Systems, Minneapolis, MN, USA), an apoptosis inhibitor, or DMSO, as a solvent control for DMSO addition in Z-VAD-FMK treated wells. Cells were infected with RSV at an MOI of 2 for one hour at 37°C. Following infection, infectious media was replaced with fresh culture media containing either 100 µM Z-VAD-FMK or 0.5% DMSO to appropriate sets of cells. Viral replication in each group was assessed 24 hpi by plaque assay.

Statistical Analysis

All statistical analysis was performed using GraphPad Prism version 6.01 (GraphPad Software, San Diego, CA, USA). Data shown are means calculated from triplicate wells with error bars representing the standard deviation (SD) unless otherwise noted. The Student *t* test was used for statistical comparisons between the experimental and control groups. When analyzing more than two groups, an analysis of variance (ANOVA) was performed and comparisons of means between each of the groups were assessed by the Tukey's multiple comparisons test.

Results

Mcl-1 knockout cells permit increased propagation of RSV in MEF cells

The replication of RSV was observed in WT and ΔMcl-1 MEF cells. MEF cells were infected at an MOI of 2 and viral titers were assessed at 12, 24, 48, and 72 hpi in both WT and ΔMcl-1 cells. Figure 3.2 displays the replication time series of RSV in each cell type as the log (PFU/ml). At each of the time points

assayed, ΔMcl-1 MEF cells displayed pronouncedly higher titers of virus. The growth was significantly different ($p < 0.001$) between WT and ΔMcl-1 cell types at each of the four time points where data was collected. ΔMcl-1 cells produced over one log more virus at each time point than WT cells and the greatest difference between replication was observed at 24 hpi when the ΔMcl-1 cells produced over 3 logs more virus than WT cells. These results indicate that the lack of Mcl-1, and inhibitor of apoptosis, enables greater replication of RSV and allows MEF cells to be more permissive to RSV infection.

Increased replication of RSV in Mcl-1 knockout cells was independent of MOI

The effect of MOI on subsequent replication in MEF cells was investigated to confirm that the previous result of increased replication of RSV in ΔMcl-1 was not due to the viral load present in the initial infection. Increased replication of RSV in ΔMcl-1 cells was shown to be independent of the number of infectious particles that were used for the initial infection of MEF cells (Figure 3.3). Both a lower and higher MOI (0.5 and 2, respectively) were used to assess any effect that the MOI of the initial infection might impart on the ensuing replication. At 24 hpi the viral titers were significantly higher ($p < .001$) in ΔMcl-1 cells than those of WT cells at the lower MOI of 0.5 and the previously tested higher MOI of 2. Replication in both the WT and ΔMcl-1 cells compared between MOIs was also significantly different. The averaged titers were around 1.5 higher in wells infected with an MOI of 2 compared to those of the same cell type infected with the lower MOI of 0.5. This result is expected, but further validates the accuracy of our selected measure of replication since observed titers increase equally between MEF cell types as infectious input (MOI) is increased. This result further confirms that the knockdown of Mcl-1 increases the ability of RSV to infect and proliferate in MEF cells.

RSV infection of MEF cells observed via confocal microscopy

MEF cells infected at an MOI of 2 were prepared for confocal microscopy to observe any apparent differences in the phenotype between infected WT and ΔMcl-1 cells. Slides were prepared for both MEF cell types at 12, 24, 48, and 72 hpi and immunofluorescently labeled to visualize RSV infected cells at each point in the time series (Figure 3.4). Cells were labeled by the nuclear counterstain DAPI (blue) while infected cells appeared green based on antibodies used to probe the cells. At 12 hpi, no difference in the phenotype of infected cells is evident between the WT and ΔMcl-1 cells; both cell types show classic infection with interspersed syncytia present. By 24 hpi, a clear and marked difference between WT and ΔMcl-1 cells can be seen. While WT cells continue to grow with expected growth patterns/cell morphology, including continued syncytia presence; the ΔMcl-1 cells displayed a pronounced increase in syncytia formation. Furthermore, the syncytia found in ΔMcl-1 slides were much larger than normal and were comprised of many more cells. At the 48 hpi time point, this trend continued as WT slides contained normally sized and dispersed syncytia while the syncytia from the ΔMcl-1 cell slides continued to be large and more numerous in comparison. At 72 hpi the continual marked difference between cell types was observed, although as expected, there were less overall cells in both MEF types due to the cytopathic effects from infection with RSV. The confocal microscopy time series analysis and micrographs highlight that ΔMcl-1 is triggering distinct and discernable phenotypic changes in how RSV replicates in MEF cells.

Confirmation of Mcl-1 knockout in ΔMcl-1 MEF cells by western blot

It was unexpected that ΔMcl-1 MEF cells would propagate RSV virus at such a higher rate than WT MEF cells. Although, the Mcl-1 knockout in MEF was confirmed by Dr. Joseph T. Opferman (St. Jude Children's Research Hospital, Memphis, TN, USA) and the generation of the ΔMcl-1 MEF mutation is

previously described (Opferman et al. 2003), we wanted to reconfirm that there was no Mcl-1 expression in ΔMcl-1 MEF cells grown in our lab and compare the Mcl-1 expression levels in murine and human RSV permissive cells. Therefore, the protein expression of Mcl-1 in the MEF cells was assayed by western blot analysis. The cell lysate for both WT and ΔMcl-1 MEF cells in addition to control lysate collected from human HeLa cells and murine RAW cells were assessed by western blot. The primary antibody used to label blots was able to detect and distinguish between both human (40 kDa) and mouse (35 kDa) Mcl-1. Western blot analysis confirmed that ΔMcl-1 was in truth a knockout cell line and Mcl-1 protein was not present at detectable levels as expected (Figure 3.5). Mcl-1 was not detected from ΔMcl-1 cell lysates but was detected for WT cell lysates at the predicted 35 kDa for mouse Mcl-1. Human HeLa cell lysates produced a band at near 40 kDa as projected for human Mcl-1 and a 35 kDa band was observed in RAW cell lysates as anticipated for a mouse cell line. A band at approximately 42 kDa, indicating β-Actin, was also detected on the blot for the 700 nm channel (red). The β-Actin protein was detected in all four of the cell lysates and clearly demonstrates that total protein added was uniform among wells. The results from the western blot analysis confirms that the Mcl-1 protein is not expressed in ΔMcl-1 MEF cells and the RSV replication results in the MEF cells are due to mutation in ΔMcl-1 cells. The expression levels of Mcl-1 in murine and human-derived cell lines were similar although the molecular weight of the protein was slightly different as expected.

Replication of Influenza virus in MEF cells

Replication of influenza virus in WT and ΔMcl-1 MEF cells was tested. MEF cells were infected with influenza A/PR/8/34 (H1N1) at an MOI of 2. Replication of the virus was assayed 72 hpi and expressed as log (PFU/ml). Like RSV, influenza virus replicated to far higher titers in ΔMcl-1 cells as

compared to WT MEF cells ($p < 0.001$) (Figure 3.6). Influenza titers were over 2 logs higher in ΔMcl-1 cells than those of WT cells. This result demonstrates that the observed phenomenon of ΔMcl-1 MEF cells' ability to produce increased quantities of virus is not limited to RSV.

Apoptosis is not upregulated in Mcl-1 knockout MEF cells until late in RSV infection

Since Mcl-1 is involved in regulating apoptosis as an antagonistic to pro-apoptotic BCL-2 family members, apoptosis activity was investigated in MEF cells. Results from our lab have identified Mcl-1 as a gene upregulated during RSV infection and Lindemans et al found that Mcl-1 was upregulated within two hours of RSV infection (Lindemans et al. 2006, Lupfer 2009). Since our results show phenotypic changes as early as 24 hpi (Figure 3.4) and increased titer in ΔMcl-1 MEF starting at 12 hpi in our time series of replication differences between MEF cells (Figure 3.2), we sought to determine the timeline for apoptosis in the MEF cell lines. Therefore, apoptotic activity was measured every 12 hours for 48 total hours in RSV infected MEF cells by caspase 3/7 activity at each point in the time series. Variations in the number of cells and background caspase activity were controlled for by performing permutation tests of the ratios of caspase activity. The measured fluorescence at 485 nm/535 nm (excitation/emission) of infected cells was divided by the caspase activity of mock-infected cells for both WT and ΔMcl-1 MEF data sets. No substantial increase in caspase activity was observed in the ΔMcl-1 MEF cells compared to WT cells until the 36 hpi measurement (Figure 3.7). After the 24 hpi time point, measured levels of caspase activity rise in ΔMcl-1 MEF wells but decline in those of WT cells. These apoptosis activity results are somewhat surprising and might indicate that the role Mcl-1 plays in infection is independent of or only in part to apoptosis, at least early in RSV infection.

Characterization of RSV replication in MEF cells with induced or suppressed apoptosis

The role of apoptosis in RSV replication in MEF cell lines was further assessed by altering apoptosis activity in the MEF cells with compounds to either induce apoptosis activity in WT cells or suppress activity in ΔMcl-1 cells. If apoptosis is a driving force behind the RSV and influenza virus replication disparity observed between MEF cell types, the addition of an apoptosis inducing compound would likely allow greater replication in WT cells. In retrospect, addition of an apoptosis suppressing compound should inhibit the replication in ΔMcl-1 cells if apoptosis modulation by Mcl-1 is causing the differential replication observed. CPT treated WT MEF cells produced significantly ($p < 0.001$) higher titers of RSV virus 24 hpi at both 2 μM and 4 μM concentrations (Figure 3.8A). CPT treatment at both concentrations induced WT cells to become more permissive to RSV infection, with both concentrations producing over seven times more virus in CPT treated WT MEF cells over control DMSO treated WT cells. CPT treatment with 2 μM produced more virus than those WT MEF cells that received 4 μM for both conditions (CPT treated and DMSO control wells). Most likely this result is due to the diverse effects of higher DMSO concentration in the wells. The effect of apoptosis suppressing Z-VAD-FMK on RSV replication in ΔMcl-1 MEF cells was assessed, but viral titers at 24 hpi with 100 μM Z-VAD-FMK were not significantly different than DMSO (0.5%) treated control ΔMcl-1 cells (Figure 3.8B). These results suggest that the role Mcl-1 plays in RSV infection is at least in part to apoptosis, but indicates that apoptosis does not completely account for the replication results observed. This implies that Mcl-1 may have an additional or synergistic, undescribed functionality that contributes to RSV's enhanced propagation in MEF cells lacking Mcl-1.

Discussion

RSV is a leading cause of respiratory infection that plays a critical role in the infectious disease burden worldwide, especially in young children. Upregulation of Mcl-1 in viral infections, including RSV, has been demonstrated (Lindemans et al. 2006, Zhong et al. 2012). Here, we show that the knockout of Mcl-1 in MEF cells allows RSV and influenza virus to replicate to much higher titers compared to WT MEF cells. This increased permissiveness was documented to increase titers as early as 12 hpi in ΔMcl-1 MEF cells. However, apoptosis, whose modulation is the recognized predominate function of Mcl-1, was not shown to be significantly upregulated until after 24 hpi. A significant increase in apoptosis in ΔMcl-1 MEF cells, measured by caspase 3/7 activity was only observed at the 36 and 72 hpi time points, the time at which WT MEF cells displayed a decline in apoptotic activity. Inducing apoptosis by camptothecin resulted in increased viral titers in WT MEF cells over DMSO solvent control WT cells, but inhibiting apoptosis in ΔMcl-1 cells did not demonstrate any significant difference in viral titer compared to control cells. Confocal microscopy revealed phenotypic changes to RSV infected cells in addition to increased titers generated in ΔMcl-1 MEF cells as ΔMcl-1 cells formed uncharacteristically large and numerous syncytia when infected. Upregulation of Mcl-1 would logically be beneficial to the virus, as a method to keep cells alive for increased viral growth and dissemination. However, our results demonstrate that knockout of Mcl-1 in MEF cells is beneficial to viral replication and therefore, upregulation of Mcl-1 during RSV infection is most likely not to the benefit of the virus.

Camptothecin (CPT) targets DNA topoisomerase I leading to a cascade ending in apoptosis (Liu et al. 2000). We used CPT to examine the role of apoptosis in the action of ΔMcl-1 inducement of RSV replication. If Mcl-1's mode of action is in apoptosis, we would expect that treatment with an

apoptosis inducer in WT MEF cells would increase replication of RSV and inversely, ΔMcl-1 MEF cells treated with an inhibitor of apoptosis should complement the loss of apoptotic inhibition found in these cells and decrease RSV replication. Our results unfortunately did not clarify or confirm the role of apoptosis in the functionality of Mcl-1 during RSV infection. CPT treated WT MEF cells were more permissive to RSV infection in our study, but apoptosis inhibition did not affect replication in ΔMcl-1 MEF cells. CPT treatment did not induce the radical change in replication noted for ΔMcl-1 MEF cells but did increase replication significantly from WT MEF cells untreated/unmodified. ΔMcl-1 MEF cells treated with Z-VAD-FMK (pan caspase inhibitor) to inhibit apoptosis exhibited no difference in replicative success for RSV from control treated ΔMcl-1 MEF cells. Conversely, in A549 cells Z-VAD-FMK promoted higher levels of RSV production (Bitko et al. 2007).

Antagonists of Mcl-1, BAX and BAK interact at the OMM to induce permeabilization and subsequent release of pro-apoptotic molecules like cytochrome c that lead to caspase activation and subsequent induction of apoptosis (Youle and Strasser 2008). However, our results indicate that apoptosis measured by caspase 3/7 activity was not induced in ΔMcl-1 MEF cells until well after replication differences between cells were observed. A previous study found similar results when apoptosis was shown to be unaffected by siRNA-mediated depletion of Mcl-1 (Minet et al. 2006). These results suggest that the function of Mcl-1 in RSV infection may be external to the predicted role of apoptosis modulation, supplementary to the apoptotic regulation, or functions in apoptosis regulation by a mechanism that eschews caspase activation of apoptosis. The key function of Mcl-1 has been defined as its anti-apoptotic regulating properties, but Mcl-1 is unique in that it has been described as having other functionality as well. Mcl-1 is essential for embryonic development, survival of several cell lineages, is implicated in

human cancers, and facilitates mitochondrial homeostasis (Perciavalle et al. 2012, Opferman et al. 2003, Rinkenberger et al. 2000, Beroukhim et al. 2010, Fleischer et al. 2006, Gui et al. 2015). It is therefore not out of the realm of possibility for Mcl-1 to modulate infection in an uncharacterized manner separate from its BCL-2 family action.

A result of interest is the phenotypic differences in MEF cells infected with RSV visualized by confocal microscopy. We observed numerous syncytia that were comprised of many more cells than WT MEF cells. The RSV fusion protein is essential for syncytia formation and induces apoptosis in the later stage of infection (Harris and Werling 2003, Eckardt-Michel et al. 2008). We also observed increased apoptosis in ΔMcl-1 cells late in infection compared to WT MEF cells. Together, these results indicate the possibility of currently unknown Mcl-1 - RSV fusion protein interactions that may account for some of the results recorded. It is possible that Mcl-1 and the fusion protein interact during infection in a way that inhibits viral replication by reducing fusion of infected cells to adjacent uninfected cells that, in the absence of Mcl-1, is left unchecked resulting in increased viral spread and subsequent enhanced viral replication. The role Mcl-1 plays in RSV infection is clearly worth further elucidation as ΔMcl-1 cells were so much more permissive to virus replication. Based on our findings in MEF cells and the early induction of Mcl-1 after RSV infection, it appears that Mcl-1 upregulation is not to benefit the virus, as the lack Mcl-1 in MEF cells resulted in significant increased replication of the virus.

Mcl-1 is a target for anti-cancer therapies (Glaser et al. 2012), making our results initially somewhat concerning in regards to the safety of using Mcl-1 as a target in such therapies. Our lab used siRNA technology to knockdown human Mcl-1 in HeLa cells but no observable replication differences were noted (see supplemental figure S3.1). Since our observed phenomenon only

occurred in MEF cells there is probably little concern for the safety of cancer therapies. However, understanding the role of Mcl-1 during RSV and other viral infections is important for the understanding of viral pathogenesis and host-viral interactions that can be exploited in an attempt to understand and curtail this important human pathogen.

Acknowledgements

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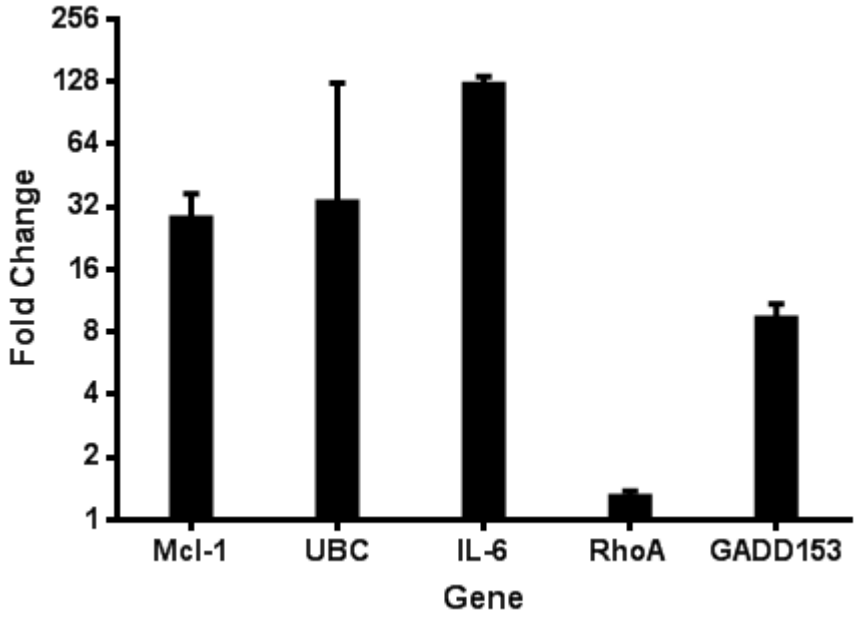


Figure 3.1. Quantitative RT-PCR verification of mRNA levels for several host genes during RSV infection. Taken from (Lupfer 2009). Microarray analysis of host gene expression during RSV infection indicated several upregulated host genes. The upregulation of these genes was confirmed using the Invitorgen SuperScript III Platinum SYBR Green One-step qRT-PCR kit from HEp-2 cells 24 hpi with RSV. Data is represented as GAPDH normalized fold change of RSV infected cells compared to uninfected controls.

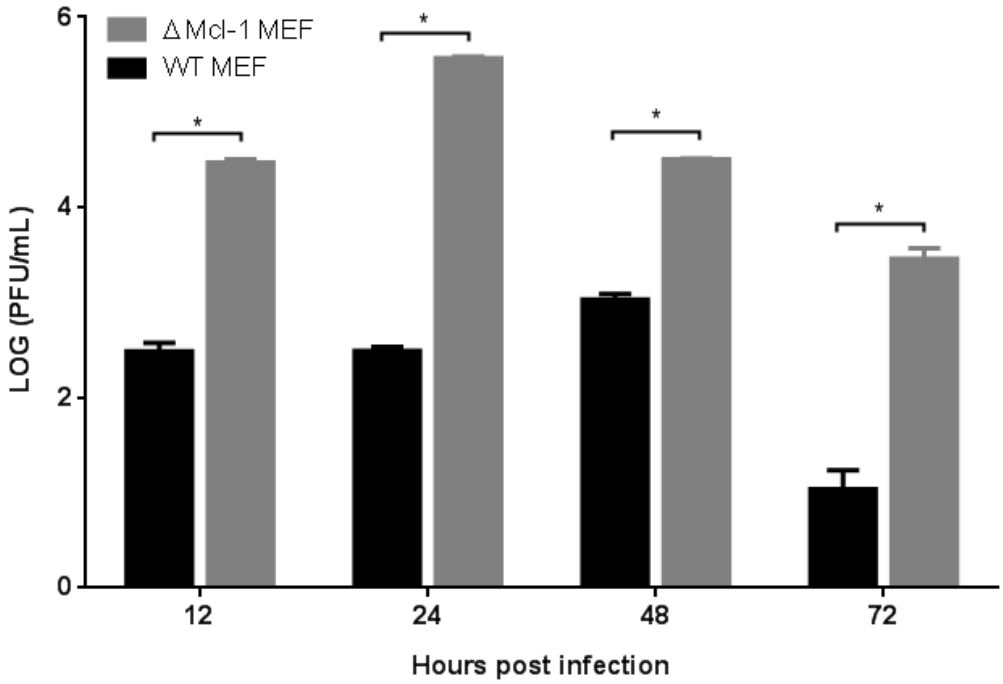


Figure 3.2. Time series of RSV replication in WT and ΔMcl-1 MEF cells. WT and ΔMcl-1 MEF cells were infected with RSV at an MOI of 2. Replication of RSV was measured after 12, 24, 48, and 72 hours post infection for each of the MEF cell lines. Viral titers were quantified by plaque assay and reported as the log (PFU/ml). Error bars represent standard deviation (SD) and significant differences indicated by asterisks ($p < 0.001$, ANOVA with Tukey post test).

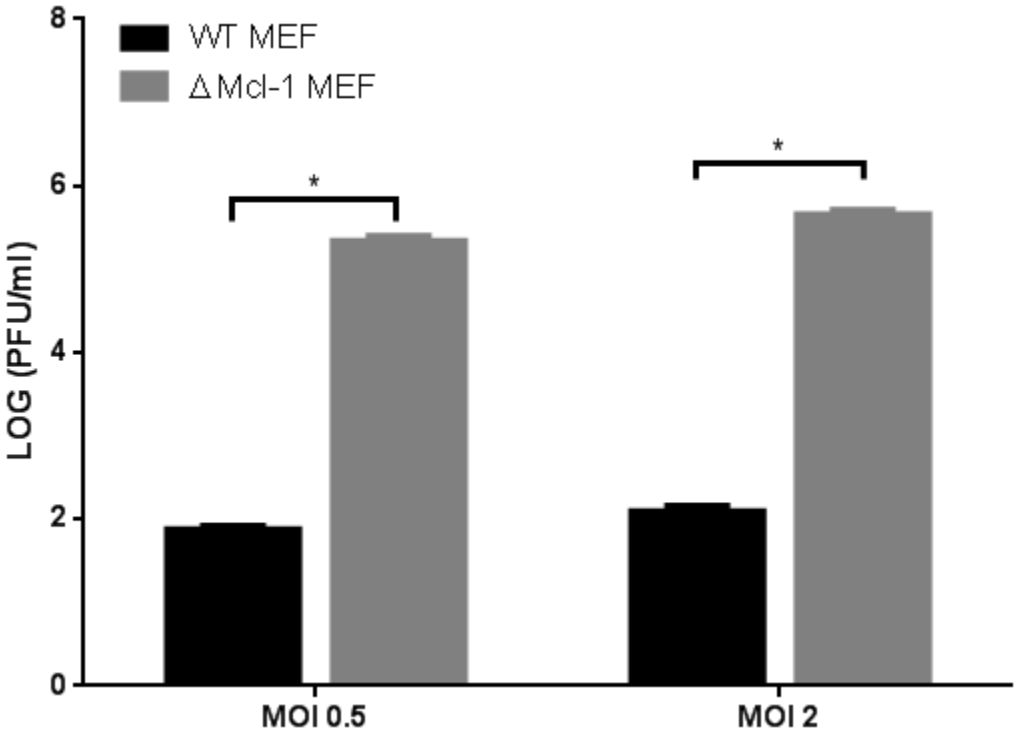


Figure 3.3. RSV replication in MEF cells. WT and ΔMcl-1 MEF cells were infected with RSV at both a high MOI of 2 and a low MOI of 0.5. RSV titers were measured by plaque assay from cell supernatants 24 hours post infection. Error bars indicate standard deviation (SD) and asterisks indicate significant differences in replication between MEF cell types ($p < 0.001$, ANOVA with Tukey post test).

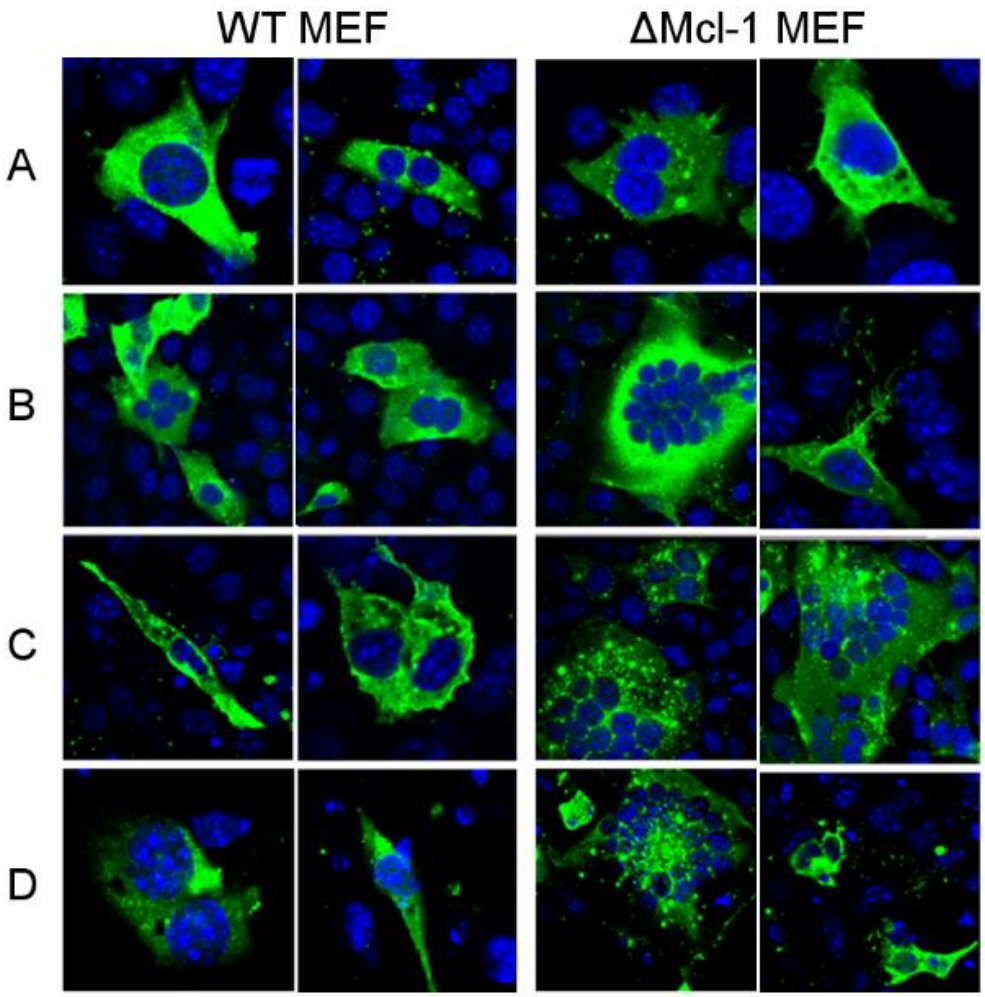


Figure 3.4. Confocal microscopy time series of RSV infection in MEF cells. At appropriate time points (12, 24, 48, and 72 hpi), MEF cells were prepared for confocal imaging; cells were stained with primary goat polyclonal anti-RSV antibody and then secondary AlexaFluor 488 rabbit anti-goat antibody. Images were acquired via confocal microscopy with a Leica Zeiss LSM510 META with Axiovert 200 motorized microscope and LSM software. Displayed are two representative micrographs each for both WT and ΔMcl-1 MEF cells infected with RSV at an MOI of 2. Horizontal rows represent hours post infection (hpi) when cells were fixed and prepared for microscopy A: 12 hpi; B: 24hpi; C: 48 hpi; D: 72 hpi. Cells were stained with DAPI (blue) and anti-RSV polyclonal antibody (green).

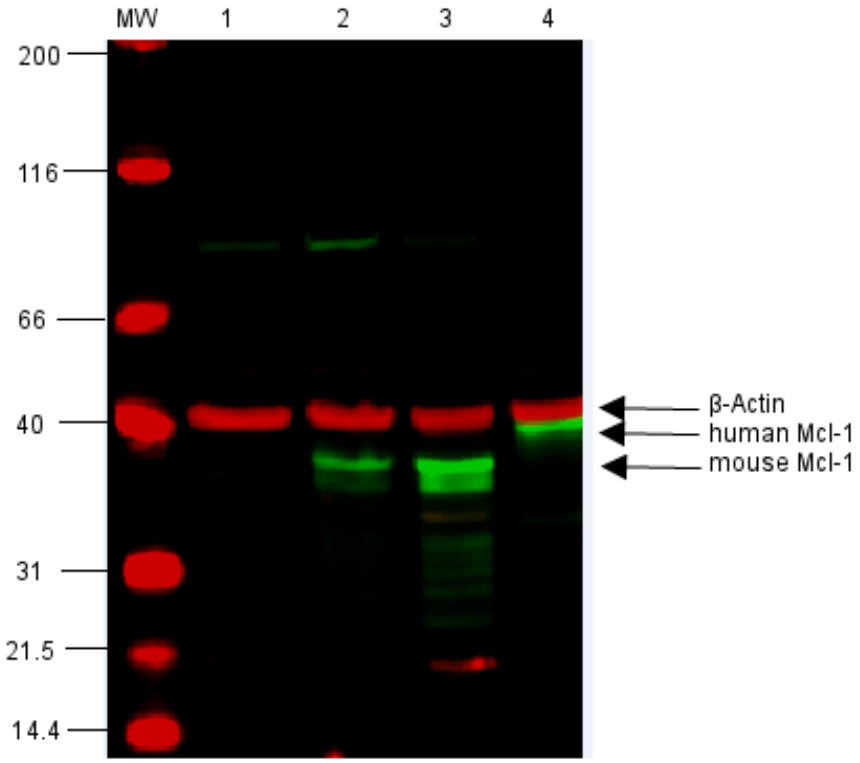


Figure 3.5. Detection of Mcl-1 protein in cell lysates by western blot. Lane MW: Prestained SDS-PAGE Standards; lane 1: ΔMcl-1 MEF cell lysate; lane 2: WT MEF cell lysate; lane 3: RAW cell lysate; lane 4: HeLa cell lysate. 800 nm channel indicates Mcl-1 protein expression in cell lysates detected by primary rabbit Mcl-1 (D35A5) antibody (Cell signaling, Danvers, MA, USA) and IRDye800 conjugated Rabbit IgG (Rockland, Pottstown, PA, USA) secondary antibody. The 700 nm channel detects the expression of β-Actin as a lysate control by primary mouse β-Actin antibody (Santa Cruz Biotechnology, Dallas TX) and secondary IRDye700 conjugated mouse antibody (Rockland).

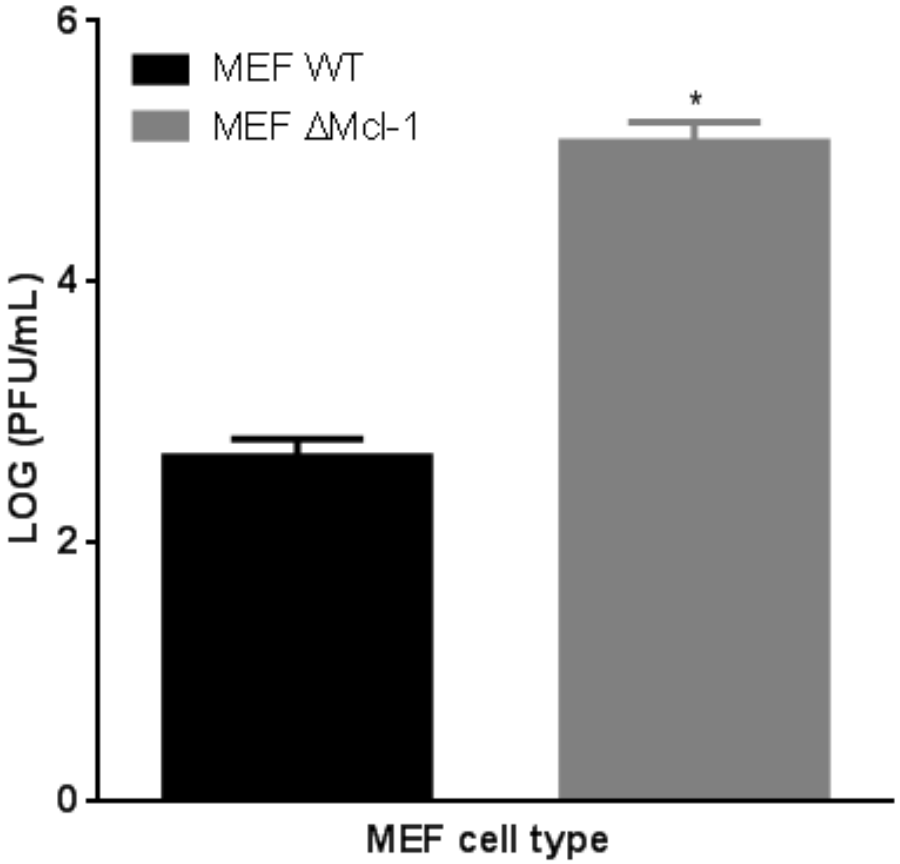


Figure 3.6. Influenza virus replication in MEF cells. WT and ΔMcl-1 MEF cells were infected with influenza virus A/PR/8/34 (H1N1) at an MOI of 2. Influenza titers were measured by plaque assay from cell supernatants 72 hours post infection. Error bars indicate standard deviation (SD) and the asterisk indicates a significant difference in replication between MEF cell types ($p < 0.001$, Student's *t* test).

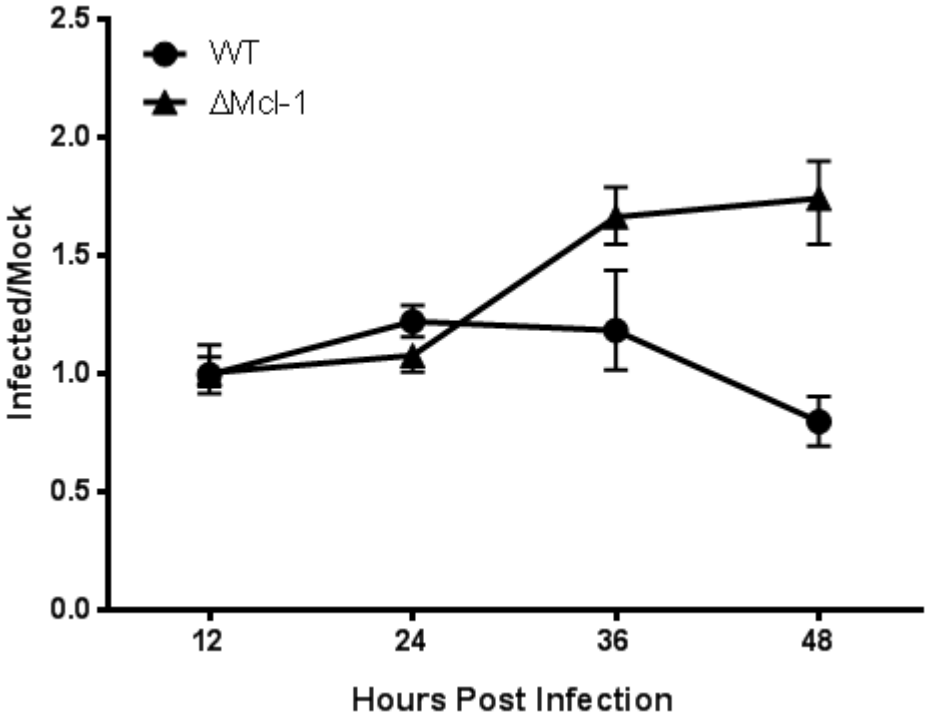
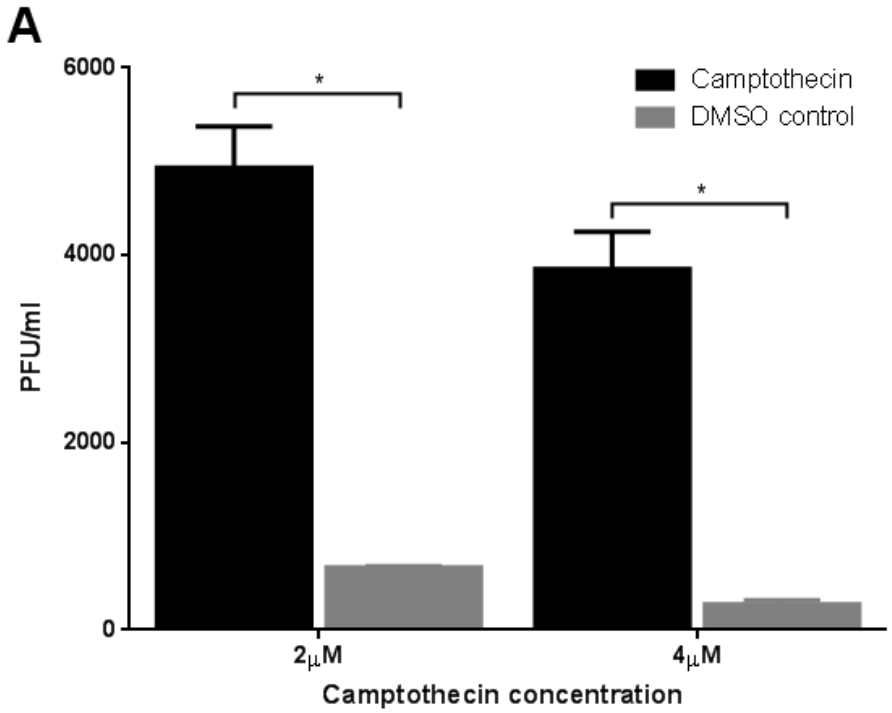


Figure 3.7. Time series of apoptosis activity in RSV infected MEF cells measured by the Apo-ONE Homogeneous Caspase-3/7 Assay. Caspase 3/7 cleavage activity was measured in both WT and ΔMcl-1 MEF cells as fluorescence at 485 nm/535 nm (excitation/emission) in triplicate wells. Apoptosis was monitored for a total of 48 hours with absorbance reading taken every 12 hours. The ratio of caspase 3/7 activity in RSV infected cells to control mock infected cells is plotted to control for background differences in caspase levels between cell types. Error bars represent 95% confidence intervals.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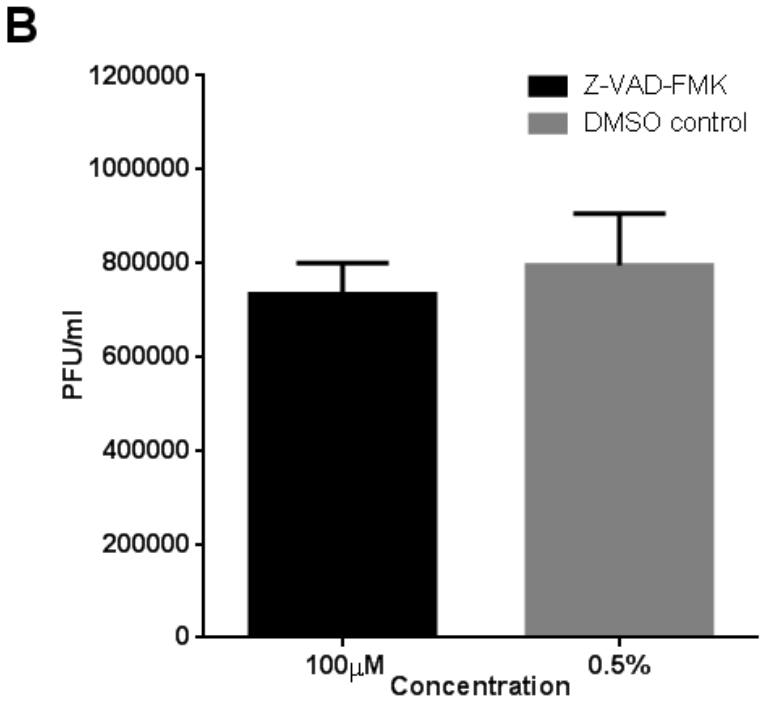
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DMSO control
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Concentration

Figure 3.8. RSV replication in MEF cells treated with apoptosis inducing or inhibiting compounds. WT MEF cells were treated with apoptosis inducing camptothecin (CPT) while ΔMcl-1 MEF cells received Z-VAD-FMK, an apoptosis inhibitor. RSV replication was measured by titration of cell culture media 24 hours post infection by plaque assay. Error bars represent standard deviation (SD) and asterisks indicate significant differences ($p < 0.001$, ANOVA with Tukey post test). A) Replication of RSV in WT MEF cells treated with 2 µM or 4 µM CPT to induce apoptosis. DMSO was added to untreated cells at the concentrations used in treated wells to control for the DMSO used as a solvent for CPT. B) Replication of RSV in ΔMcl-1 MEF cells treated with 100 µM Z-VAD-FMK. DMSO was added to otherwise untreated cells to control for the concentration added by treatment with Z-VAD-FMK.

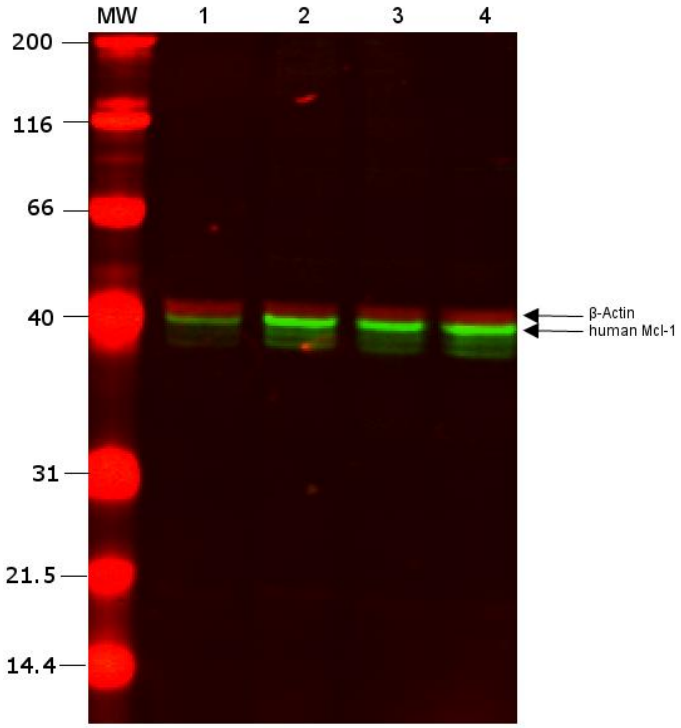

Figure S3.1. Detection of Mcl-1 protein expression in siRNA treated HeLa cells by western blot. HeLa cell lysates were analyzed for Mcl-1 expression to confirm knockdown of Mcl-1 using siRNA specific to human Mcl-1 (Cell Signaling, Danvers, MA, USA). A control-siRNA and a transfection control cell lysate were collected as treatment controls and a media mock treatment as a baseline control for Mcl-1 expression in HeLa cells. Lane MW: Prestained SDS-PAGE Standards; lane 1: Mcl-1-siRNA HeLa cell lysate; lane 2: control-siRNA HeLa cell lysate; lane 3: transfection control HeLa cell lysate; lane 4: HeLa cell lysate. The 800 nm channel indicates Mcl-1 protein expression in cell lysates detected by primary rabbit Mcl-1 (D35A5) antibody (Cell Signaling) and IRDye800 conjugated Rabbit IgG (Rockland, Pottstown, PA, USa) secondary antibody. The 700 nm channel detects the expression of β-Actin as a lysate control by primary mouse β-Actin antibody (Santa Cruz Biotechnology, Dallas TX, USA) and secondary IRDye700 conjugated mouse antibody (Rockland). No replication differences were observed by plaque assay (data not shown).

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Chapter 4. A rapid diagnostic assay for dengue virus type 2 using recombinase polymerase amplification

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Abstract:
Dengue virus (DENV) is the most rapidly spreading arbovirus worldwide with well over one-third of the world's population living in areas at risk for contracting the virus. Early and rapid detection of dengue is crucial to lessening the burden of this significant pathogen. Early diagnosis allows for patients to receive appropriate care reducing the drain to health systems and the danger of progression to more severe disease. Here we describe the use of reverse transcription recombinase polymerase amplification (RT-RPA) to detect dengue virus type 2 (DENV2). The DENV2 RT-RPA assay is a sensitive and specific isothermal reaction capable of differential detection of DENV2 in less than 20 minutes with a sensitivity as low as 50 copies. We display the use of two detection formats to view RT-RPA products and highlight the dynamic nature of the RT-RPA assay. Here we demonstrate that our DENV2 specific RT-RPA assay is a sensitive, quick, and adaptable alternative, suitable for early detection of DENV2.

Introduction
Dengue virus (DENV) is a significant and widely disseminated virus of the *Flaviviridae* family. The World Health Organization (WHO) defines dengue as a global pandemic threat and the most important mosquito-borne viral disease worldwide (WHO 2012). Dengue is endemic in over 100 countries in tropical and subtropical locales but it is also the source of more sporadic outbreaks in even more sites worldwide (WHO 2012). There are predicted to be anywhere from 100 to 390 million new infections per year, and alarmingly, the incidence of dengue has increased 30-fold within the last five decades (Bhatt et al. 2013, Organization 2012). Outbreaks of infection exert a costly burden, from the health systems and economy of the affected populations, to the mortality and morbidity of individuals; underreporting of disease along with misclassification has also been documented (Bhatt et al. 2013, Shepard,

Undurraga, and Halasa 2013, Edillo et al. 2015, Gulland 2013, Salmon-Mulanovich et al. 2015, Shepard et al. 2014, Undurraga et al. 2015, Wichmann et al. 2011, Beatty et al. 2011). Management of dengue is a current and increasing problem, as symptoms can mimic other endemic diseases (Potts and Rothman 2008). This leads to strain on health systems and ineffectual treatment without diagnostic tests that are rapid, sensitive, and can be used soon after symptoms develop.

Symptoms of disease range from asymptomatic infection to an assortment of clinical presentations. Most common is the milder dengue fever (DF) but a small portion will progress to more severe and possibly fatal dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (Whitehorn and Simmons 2011). There are four serotypes of the virus (DENV1-4). While primary infection results in immunity against reinfection to a particular serotype, individuals are still susceptible to infection by the remaining serotypes. Secondary infection generally leads to more severe disease due to antibody-dependent enhancement (ADE), but the specific sequence of serotype infection has also been reported to play a role in severity (Guzman, Alvarez, and Halstead 2013, Fried et al. 2010, Endy et al. 2004, Anantapreecha et al. 2005, de Araújo et al. 2009, Gibbons et al. 2007, Halstead 2006, Sangkawibha et al. 1984, Thomas et al. 2008, Vaughn et al. 2000).

Proficient and accurate diagnosis of DENV is paramount, affecting a multitude of areas critical to DENV management including DENV surveillance as well as clinical care issues such as early detection, case validation, and differential diagnosis from other diseases (WHO 2009). A number of methods are employed to detect the dengue virus, depending on the situation. Early after onset (4-5 days) the virus itself can be detected, but following this phase, serology must be employed as virus is no longer detectable. In general, tests

with high confidence tend to be more complex and time consuming leading to their infrequent use while faster, widely accessible and heavily employed serological based tests (relying on antibodies) tend to be less sensitive or cannot be used for early detection (WHO 2009).

Viral isolation is the reference method, but is time consuming and not a viable option for identification in a clinical setting (Yamada et al. 2002, Medina et al. 2012). Serology based tests that measure IgM and IgG by enzyme-linked immunosorbent assay (ELISA) as well as antigen detection from the nonstructural protein 1 (NS1) are widely used in clinical settings for their speed, but sensitivity and specificity are diminished (Hunsperger et al. 2014, Peeling et al. 2010, Hunsperger et al. 2009, Fernández and Vázquez 1990, Moi et al. 2013, Paranavitane et al. 2014, Blacksell et al. 2012, Felix et al. 2012, Saito et al. 2015). Nucleic acid detection allows for the detection of the virus in a specific and relatively quick timeframe. Real-time RT-PCR (Lanciotti et al. 1992, Johnson, Russell, and Lanciotti 2005, Najioullah, Viron, and Césaire 2014, Santiago et al. 2013) has been utilized for many years but is not conducive to point-of-care testing. Recently, isothermal amplification techniques like nucleic acid sequence based amplification (NASBA) (Wu et al. 2001) and reverse transcription loop mediated isothermal amplification (RT-LAMP) (Sahni et al. 2013, Teoh et al. 2013) have been described as an alternative method for DENV nucleic acid detection that keep sensitivity while improving accessibility.

Recombinase polymerase amplification (RPA) is an advantageous isothermal amplification technique to more traditional PCR based detection strategies as well as compared to other isothermal detection approaches. RPA is an extremely quick (≤20 minutes) DNA amplification method that exploits three critical enzymes to produce products. Two opposing specific oligonucleotide primers form a complex with recombinases targeting them to their

homologous template sequence (Piepenburg et al. 2006). Single stranded binding protein (SSB) then bind to the displaced template DNA strands and DNA synthesis proceeds via a strand displacing polymerase (Piepenburg et al. 2006). Reactions that contain reverse transcriptase (RT) enzyme can be utilized so that RNA templates are first converted to cDNA which is then amplified in a single tube. The products can be detected by real-time fluorescence detection or by a colorimetric signal on a simple lateral flow device (LFD). Real-time RT-RPA is accomplished by the addition of a fluorescent exo-probe to the reaction mix while LFD detection is accomplished by the substitution of a 5' modified primer set followed by the application of diluted product onto the LFD. Here we describe the development of a RT-RPA method for the detection of the DENV2 serotype.

Materials and Methods

Virus and RNA

DENV2 laboratory strains were grown in Vero cells, harvested, and stored at -80°C. Laboratory strains of DENV2 used in this study were NGC, S221, and TH-36. RNA collected from the laboratory strains was extracted for RT-RPA using the NucleoSpin RNA isolation kit (Macherey-Nagel, Düren, Germany). Extractions were performed in accordance to supplied protocols and guidelines, and RNA was aliquoted into single use tubes then stored at -80°C.

Primer and exo-probe design

Primers compatible to the RPA reaction were designed to specifically amplify DENV2. The primer set was designed to amplify a 316 bp segment of the DENV2 viral genome corresponding to a portion of the structural membrane (M) protein. Real time RT-RPA was conducted using the standard unmodified opposing primers and the addition of an exo probe with the fluorescent dye carboxyfluorescein (FAM) (Table 4.1) in the RPA reaction. The TwistAmp exo probe has several modifications that allow for the accurate and robust

monitoring of RPA reactions. The exo probe contains a tetrahydrofuran (THF) residue which replaces a nucleotide in the target sequence acting as a substrate for the DNA repair enzyme Exonuclease III. The THF residue is flanked by base replacements of a dT-fluorophore and a corresponding dT-Black Hole Quencher (BHQ). In the presence of amplicon, the probe is annealed to the target sequence allowing the exonuclease to cleave the probe at the THF position. This results in the separation of the fluorophore and the quencher leading to a generation of fluorescent signal and the ability to observe the accrual of specific amplicon. A 3' modification inhibits any unwanted amplification of the exo probe itself (TwistDx). LFD detection of amplification products was attained by the substitution of the basic opposing RPA primers for the pair of 5' modified primers (Table 4.1) in the RT-RPA reaction.

RT-RPA

The amplification of DENV2 genome was accomplished by the use of the TwistAmp exo RT kit (TwistDx, Ltd., Cambridge, United Kingdom). Dependent on the detection format chosen, appropriate primers were used in the reaction (Table 4.1). The kit contained a lyophilized pellet containing reaction enzymes including the necessary RT enzyme to generate complementary DNA (cDNA) from an RNA template, premeasured and distributed by the manufacturer. The reaction pellet was dissolved according to the manufacturer's instructions using a mix of rehydration buffer, diluted template, appropriate primer or primer/probe sets for visualization format selected, and water to a final reaction volume of 47.5 µl. Magnesium acetate (280 nM) was used to initiate the RPA reaction as described in the manufacturer's instructions bringing the final volume to 50 µl. A negative control (water with no DNA) sample was included in each run along with a positive control using NGC DENV2 for assay runs with clinical samples. The reaction was conducted at 40°C (unless

otherwise specified) for 20 minutes and amplification products observed by LFD or real-time fluorescence as indicated.

Confirmation of RT-RPA amplified DENV2 DNA product.

To confirm the correct DNA product was amplified, Sanger sequencing was performed at the Center for Genome Research and Biocomputing (CGRB) at Oregon State University. Following amplification, products were purified using a GeneJET DNA purification kit (Thermo Scientific, Waltham, MA, USA) and samples were observed by gel electrophoresis on a 1.5% agarose gel stained with SYBR Safe (Life Technologies, Carlsbad, CA, USA). DNA was visualized on an LED blue light transilluminator and the band corresponding to the approximated product size (316 bp) was excised using a razor blade and DNA purified using the GeneJet kit. Sequencing results were aligned to the predicted DENV2 sequence by the EMBOSS Water pairwise alignment tool.

Fluorescence detection of Real time RT-RPA:

Real-time RT-RPA was achieved by using the TwistAmp exo RT kit (TwistDx). Reactions containing the basic RPA primers with the addition of the DENV2 specific exo-probe allowed for amplification products to be visualized in real time by observing the fluorescent signal. The amplification was monitored using the portable Twista real-time fluorometer (TwistDx) or, in a small fraction of experiments involving temperature gradient that the Twista is unable to perform, the Bio-Rad CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) was used to detect fluorescence.

Lateral Flow Device detection of RT-RPA products:

Rapid detection of the DENV2 genome was also achieved by LFD. DENV2 genome was first amplified using the TwistAmp exo kit (TwistDx) with a substitution of the 5’ modified primers into the reaction mix. End products, dually labeled with biotin and FAM, were then diluted with the supplied buffer

and applied to the commercially available LFD, PCRD-2 (Forsite Diagnostics, United Kingdom). For the LFD, a colorimetric signal indicates the presence of doubly labeled DNA at the test line (T-line) via immobilized anti-FAM and anti-biotin antibodies and a control line (C-line) is incorporated as a control to check for appropriate flow. Positive results are displayed by the appearance of two lines, one each at the C and T-line positions. Negative results are indicated by a single line at the C-line positon and tests that do not display any lines are considered faulty and therefore unusable. The use of the LFD for detection of RPA products added no more than 10 minutes to the already quick overall reaction time and allowed for visualization of product without the costly addition of the exo-probe and machinery necessary for the real-time assay format.

Indonesian patient samples

The RT-RPA assay was performed on a small subset of clinical samples collected from patients at the Hospital Dokter Hasan Sadikin (Bandung, Indonesia) and processed at the Medical Faculty University Padjajaran (Bandung, Indonesia). Two samples (DENV_4, DENV_5) represent RNA extracted from cultured DENV2 collected from patients diagnosed positive and confirmed by Lanciotti (RT-PCR) (Lanciotti et al. 1992). In addition, RNA from three patient serum samples (HTV093, HTV277, and HTV201) suspected to be DENV positive but previously unconfirmed were extracted using a Qiagen RNA extraction kit and tested by our DENV2 specific RT-RPA real time assay.

Watman FTA® card sample collection

The purpose of using FTA cards for sample collection is two-fold. First, people in developing countries who live in remote villages may not be able to travel to city hospitals and the FTA card may benefit them if they are willing to send a finger pin-prick blood sample on FTA card for diagnosis. Secondly,

surveillance of mosquitoes for dengue from different locations can be accomplished as the FTA card preserves RNA samples for a considerable time until processing.

Sample collection using Whatman FTA® technology (GE Healthcare Bio-Sciences Corp., USA) was also tested. Initial screens were prepared using spiked samples that contained 100 µl of DENV2 (NGC) virus collected from cell culture per card. Virus was added to an Indicating FTA® Micro Card allowed to dry for a minimum of 2 hours, then processed. Clinical samples were also collected from patients from M.S. Ramaiah Medical College Hospital (Bangalore, India). For each of the nine patients sampled, both whole blood and serum samples were collected and applied to FTA® cards. These samples were confirmed by hospital personnel to have DENV infection by the NS1 based ELISA method (Dengue NS1 Ag Microlisa Kit, J.Mitra and Co Pvt. Ltd, India) but no information on serotype was available. Cards were stored for several months at room temperature followed by RNA extraction from the FTA® cards by the Molecular Diagnostics Laboratory at Oregon State University. Sample preparation was accomplished using the Ambion RNA Rapid Extraction solution (Life Technologies) followed by isolation using the Ambion MagMAX 96 viral RNA isolation kit (Life Technologies). The undiluted RNA was used as template in the RT-RPA assay and reactions were run in duplicate.

Results

Sensitivity of DENV2 detection by real-time RT-RPA

The sensitivity of the RT-RPA assay was determined by the real-time detection format. DENV2 (NGC) template was diluted to 100, 50, 25, and 10 copies per RPA reaction. Ten replicates of each dilution were evaluated and a negative control using nanopure water was incorporated into each set of

reactions analyzed on the Twista fluorometer. Table 4.2 displays the number of positive replicates for each dilution tested. While the RPA assay was capable of amplifying DENV2 at lower levels, reactions containing 50 copies per reaction were the lowest dilution tested where all replicates produced distinct amplification products within the 20 minute reaction time. In addition, our DENV2 specific primer set was evaluated for its efficacy in detecting additional strains of DENV2 RNA. A minimum of three replicates for each strain were tested and RNA was diluted to 10^{-2} in nuclease free water. The RT-RPA assay was able to amplify each of the strains tested (Figure 4.1, Table 4.3). The resulting amplification products can be seen in the representative image of Figure 4.1. These data demonstrate that our real-time detection format for detection is sensitive to DENV2.

Specificity of RT-RPA using real-time detection

To test how specific the RT-RPA assay was to DENV2, a number of templates were examined with a minimum of three replicates each. Two negative template controls were surveyed: water as template and a tissue culture negative control. Furthermore, several RNA templates expected to be negative based on primer design were also evaluated. These included a number of non-related viral templates: respiratory syncytial virus (RSV), influenza (PR/8/34), and rabbit herpes virus, as well as several related flavivirus members; West Nile virus (WNV) and RNA from the other DENV serotypes. Table 4.3 summarizes the results of the specificity testing for our RT-RPA assay using real-time detection and indicate that the primers selected for our RT-RPA assay are specific to DENV2. Sequencing of products was performed for NGC samples as described in methods and confirmed that RPA product generated by the RT-RPA assay using the DENV2 specific primers resulted in amplifying the DENV2 sequence predicted.

LFD detection of RT-RPA products

Additionally, DENV2 products amplified by our RT-RPA assay were also detected by LFD (Figure 4.2). Positive signal was visualized for each of the DENV2 strains tested with the LFD format for visualization of RT-RPA products, demonstrating the versatile nature of the RT-RPA assay.

Assessment of DENV2 specific RT-RPA assay in clinical samples

The DENV2 specific RT-RPA assay was further assessed in several ways. A small subset of clinical based samples was used to investigate the efficacy of the assay: two samples (DENV_4, DENV_5) from confirmed DENV2 infected patient samples grown in cell culture and three (HTV201, HTV277, HTV337) RNA samples extracted from blood that were collected from patients suspected to have active DENV2 infections. Both cell culture-based samples taken from confirmed DENV2 infected patients resulted in amplification using our RT-RPA assay (Table 4.3). However, all three of the samples taken from blood samples produced no signal (Table 4.3). These three were later typed to be DENV3 virus by RT-PCR, further corroborating the specificity of our primer set.

RNA collected from FTA® cards spiked with NGC DENV2 was amplified by our RT-RPA assay and visualized using both real-time and LFD formats. Following this spiked trial, the clinical samples obtained from patients in India were tested. RNA from both whole blood and serum FTA® card samples were analyzed by RT-RPA specific to DENV2. For each of the replicates tested, one of the patient serum samples (PK520812s) was amplified by RT-RPA measured by both the real time and LFD formats. The corresponding whole blood sample from the same patient did not amplify, nor did any replicate for the other patient samples collected from the FTA® cards. We believe more work is needed to accomplish a positive reaction from FTA blood samples and to establish a modified protocol for FTA blood samples. These results

indicate that the RT-RPA assay can amplify DENV2 genome from a small amount of sample and the use of the FTA® card is a viable option for safe, storable collection of serum samples. Further, FTA cards can be used for sample transport from patients located in remote villages and for surveillance of mosquitoes for dengue virus.

Assessment of DENV2 specific RT-RPA assay reaction conditions

Reaction conditions were also examined for the RT-RPA assay. Using the real-time format, identical RT-RPA reactions were run under several different temperature conditions. The Twista reader is capable of holding the reactions at only a single temperature, therefore the CFX thermocycler was used as a fluorometer so samples could be subjected to a gradient of constant temperatures. Reactions contained 10^{-2} dilution of DENV2 (NGC) and the temperatures tested ranged from the manufacture's recommended 40°C down to 33°C. As seen in Figure 4.3, each temperature tested resulted in amplification products, highlighting the adaptable nature of RPA amplification in regards to temperature.

Discussion

Rapid and sensitive detection of DENV is critical to the disease management of a virus with such a prominent burden to human health. Here, we describe the use of RT-RPA to detect DENV2 genomic RNA and exhibit the assay's appeal as an alternative method of detection that is fast, reliable, adaptable, and easy to perform without the use of expensive machinery or advanced technical skillset. We demonstrate that our DENV2 specific assay is sensitive, as it is able to amplify DENV2 with as little as 50 copies per reaction, and was able to amplify both laboratory and clinical isolates/strains. The assay is also specific for DENV2, allowing for serotype differentiation in addition to reliably amplifying only samples containing DENV2 genomic template. Additionally,

we show that the assay is versatile given that products can be visualized by both LFD and real time formats, and that altering the assay temperature has little effect on the success of amplification.

RPA is among a number of newly developed techniques to amplify nucleic acid that retains the sensitivity associated with PCR strategies while decreasing amplification time, and has been increasingly reported for use in the detection of a number of pathogens (Abd El Wahed, El-Deeb, et al. 2013, Abd El Wahed, Patel, et al. 2013, Ahmed, van der Linden, and Hartskeerl 2014, Amer et al. 2013, Boyle et al. 2013, Boyle et al. 2014, Euler, Wang, Otto, et al. 2012, Euler, Wang, Nentwich, et al. 2012, Xia et al. 2014, Xia et al. 2015, Jaroenram and Owens 2014, Mekuria, Zhang, and Eastwell 2014, Tsaloglou et al. 2015, Chao et al. 2015, Yehia et al. 2015, Abd El Wahed et al. 2015, Escadafal et al. 2014, Kersting et al. 2014). The RPA reactions are performed in a single tube format with little manipulation or technical skill required for amplification. Reaction times are within 20 minutes, dependent on the amount of template added, and the technology has been shown to be suitable for resource limited settings critical to point-of-care detection (Escadafal et al. 2014, Crannell, Rohrman, and Richards-Kortum 2014, Lutz et al. 2010).

Our RPA assay is both sensitive and quick with a reaction time of ≤ 20 minutes. Compared to other methods for dengue detection our DENV2 specific RPA assay is equally or more sensitive in amplifying DENV2 genome in less time with little to no expensive technology required. While our subset of clinical samples from Indonesia was small both of the DENV2 positive samples were detected by our RT-RPA assay and none of the later confirmed DENV3 clinical samples produced signal with our assay. DENV3 has been reported as the predominant serotype in Indonesia so these results are

unsurprising (Suwandono et al. 2006, Corwin et al. 2001, Gubler et al. 1979, Ong et al. 2008).

Further analysis with larger subset of samples is needed to further corroborate these results with more clinical strains. Additionally, different primers and probe sets or manipulation of reaction parameters such as primer and magnesium acetate concentration and reaction temperature, could further improve the sensitivity, although testing these modifications is beyond the scope of this study. We demonstrate that the reaction temperature has little effect on reactions producing amplification products, but there is an ideal temperature for optimal enzymatic performance. As seen in Figure 4.3, a slight delay was observed for the kinetics of the amplification for reactions run below 35°C. We would predict that further cooling the reactions would lead to more pronounced shift in the time amplification products are seen, possibly affecting low template samples, therefore reaction should be run as close to 40°C as possible. The ability of RPA to work at a wide range of temperatures highlights its robust nature, one of its attributes as a detection strategy.

A simplified assay with less expensive machinery is ideal, especially for dengue where testing may occur in more resource limited settings. As point-of-care testing is ideal, we tested both real-time and LFD detection methods because each has advantages depending on the needs of the user and setting and/or situation where dengue testing may occur. With the real-time detection format, product can be measured by some existing real-time PCR detection systems (alterations made to how the device takes readings) so new equipment wouldn't necessarily be needed. However, the Twista is much less expensive to purchase and has the ability to be taken to remote locations to act as a more point-of-care test. As a downside, there is a cost to the fluorometers and the probe needed is also costly compared to more simple probes or primers required by LFD. The LFD is beneficial as a costly probe is

not needed and with less than ten additional minutes added to the amplification time a colorimetric signal indicates the presence of products without any machinery. LFD strips could also be manufactured that allow for multiplexing and eventual diagnosis of DENV1-4 on a single strip as other differential diagnosis has been performed by LFD (Lee et al. 2015, Song et al. 2014). On the downside, LFD strips are an additional consumable and can suffer from false-negative and positive signals (Pike, Godbert, and Johnson 2013, Hu et al. 2014).

The FTA® card is a filter paper based system to collect and archive nucleic acids. The cards lyse and neutralize biohazards allowing for safe collection of nucleic acids in addition to storage of samples at room temperature (Ahmed et al. 2011, Picard-Meyer, Barrat, and Cliquet 2007, Rogers and Burgoyne 1997). The use of FTA® cards to collect serum samples from patients would allow for easy transport and storage of samples before processing and detection by the RT-RPA assay. One serum sample collected from a patient with confirmed DENV was amplified by our RT-RPA DENV2 assay. It could be the other samples were from patients infected with a different serotype, or the amount collected and harvested by the FTA® card was insufficient for amplification. Serotyping of the other samples could not be performed. Further observation of the limit of detection using the FTA® card is needed for the future. However, the ability of samples collected on FTA® cards to be amplified by our RPA assay was confirmed both empirically with spiked samples as well as with a clinical patient samples using serum. The use of the FTA® card further corroborates the sensitivity of our assay as the cards only hold a small sample and even more nucleic acid is lost through processing.

While our DENV2 RT-RPA assay is specific to DENV2 and is capable of amplifying only one serotype, the results show that RPA is a contender for early detection of DENV and our lab is working on primer sets specific to the remaining serotypes. Distinguishing between serotypes is not at the forefront of concern for early diagnostics which aim at recognizing DENV before early warning signs of severe diseases and for early differential diagnosis from other diseases with similar clinical symptoms common to areas dengue is endemic. However, differentiating between serotypes would be ideal and single tube differential amplification by RPA is theoretically possible by multiplexing and detection with either format.

RPA is an attractive alternative for the rapid and early diagnosis of DENV virus. RPA is fast, reliable, and adaptable. The assay has the potential to become a sensitive tool in early point-of-care detection of DENV and we believe warrants further development for the use in clinical applications.

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Table 4.1. RPA primers used for the rapid detection of DENV2.

Name	Sequence 5'-3'
Real-time	
DENV2_3F	ACCTTGGTGAATTGTGTGAAGACACAATCACG
DENV2_1R	CCTATGGTGTATGCCAGGATTGCTGCCATTATGGT
DENV2_Probe	ATGGGACTGGAGACACGAACTGAAACA[**dT(FAM**)]G[**THF**]A[**dT(BHQ-1**)]GTCATCAGAAGGGG
LFD	
DENV2_3F_LFD	**FAM**-ACCTTGGTGAATTGTGTGAAGACACAATCACG
DENV2_1R_LFD	**Biotin**-CCTATGGTGTATGCCAGGATTGCTGCCATTATGGT

Bold text within the sequence denotes modifications made to the oligonucleotides. dT(FAM): thymidine nucleotide bearing fluorescein, THF: tetrahydrofuran spacer replacing G nucleotide, and dT(BHQ-1): thymidine nucleotide bearing the black hole quencher 1.

Table 4.2. RPA amplification of DENV2 measured by real-time detection using FAM fluorescent signal obtained on the Twista fluorometer.

Copies of DENV2 per reaction	Positive amplification
100	10/10
50	10/10
25	8/10
10	9/10

Table 4.3. Summary of DENV2 RPA amplification using real-time detection on the Twista fluorometer. The source of the template used in the RT-RPA reaction is indicated in the template column and the amplification column indicates whether amplification was observed.

Template	RT-RPA amplification
Laboratory samples	
DENV2: strain NGC	Positive
DENV2: strain TH-36	Positive
DENV2: strain S221	Positive
DENV1: strain Westpac	Negative
DENV1: strain TH-SMAN	Negative
DENV3: Strain H87	Negative
DENV4: Strain H241	Negative
DENV4: Strain TVP 360	Negative
WNV	Negative
RSV	Negative
Influenza	Negative
Herpes virus	Negative
Tissue culture negative	Negative
negative control (water)	Negative
Clinical samples	
DENV_4	Positive
DENV_5	Positive
HTV 201	Negative
HTV 277	Negative
HTV 337	Negative

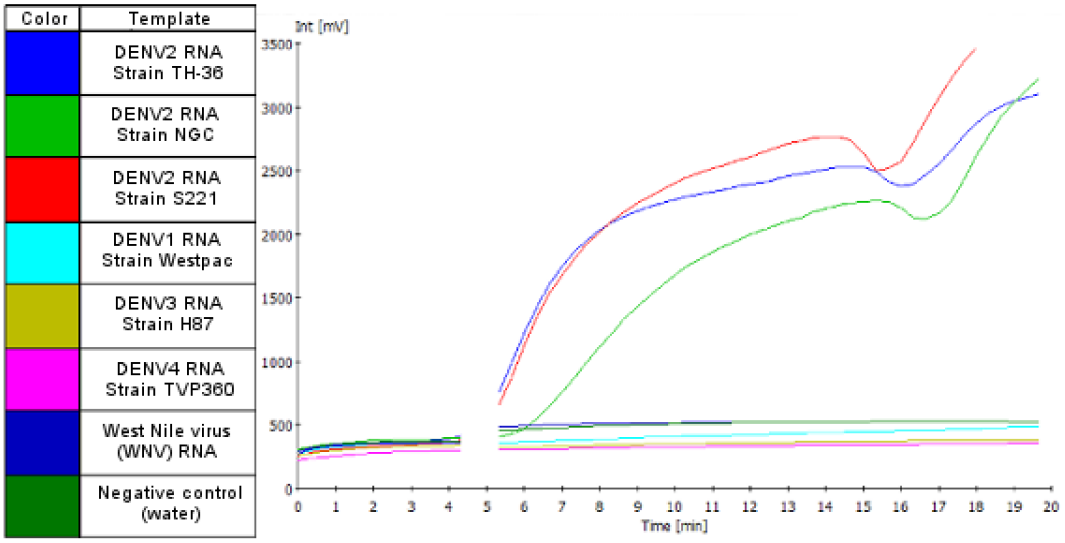

Figure 4.1 A representative image of real-time RT-RPA amplification products using the Twista fluorometer. The graph depicts the fluorescent signal for each template used in the RT-RPA reactions and the key to the left indicates the template each color represents. At approximately five minutes into run time a required mixing step was performed resulting in signal not being generated for the duration of that step.

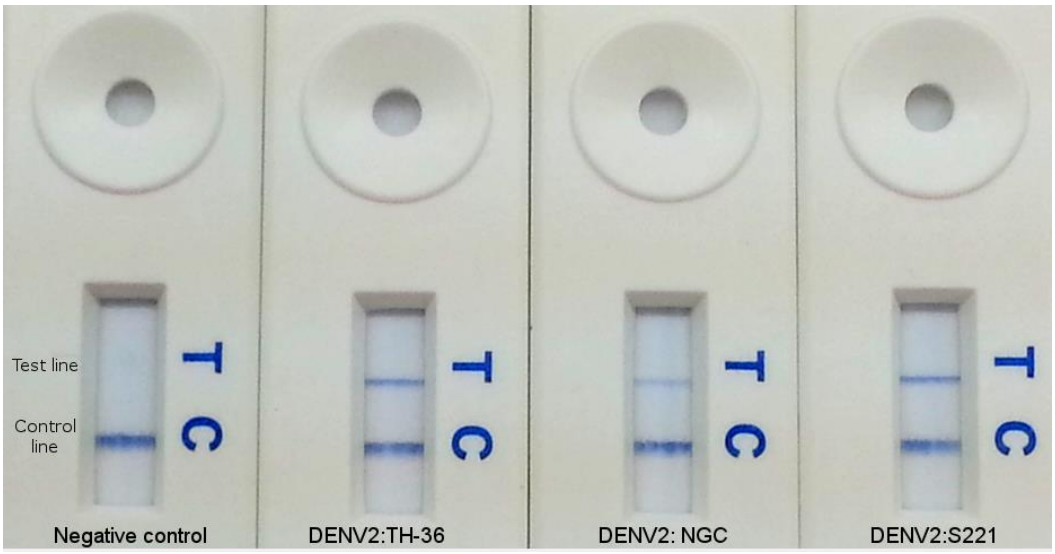

Figure 4.2. Amplification products for DENV2 RT-RPA assay visualized by LFD. Colorimetric signal at the test line (T) indicates positive amplification. Signal at control line (C) indicates a valid test.

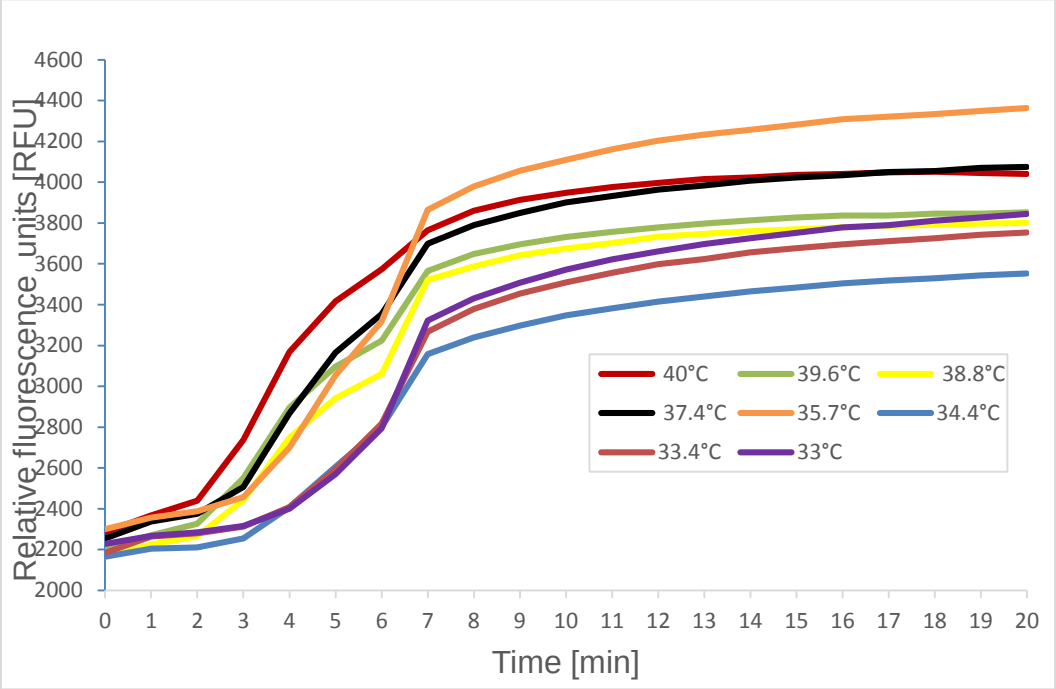

Figure 4.3. A representative image of the reaction temperature range for DENV2 specific real-time RT-RPA amplification products. Fluorescence measurements were made using the CFX96 Touch™ Real-Time PCR Detection System. The graph depicts the fluorescent signal for each of RT-RPA reactions which varied only in the temperature conditions. The key indicates the color on the graph that represents the temperature each RT-RPA reaction was run under.

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Chapter 5. Rapid and Sensitive Detection of Cyprinid Herpesvirus 3 in Latently Infected Koi (*Cyprinus carpio*) by Recombinase Polymerase Amplification

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Abstract

Since the emergence of cyprinid herpes virus 3 (CyHV-3), outbreaks have been devastating to koi and common carp leading to high economic losses. Current diagnostics for detecting CyHV-3 are limited in sensitivity and are further complicated by latency. Here we describe the detection of CyHV-3 by recombinase polymerase amplification (RPA). The RPA assay can detect as low as 10 copies of CyHV-3 genome by an isothermal reaction and yields results in approximately 20 minutes. Using the RPA assay, CyHV-3 genome can be detected in total DNA of white blood cells isolated from koi latently infected with CyHV-3, while only less than 10% of the latently infected koi can be detected by a real-time PCR assay. In addition, RPA products can be detected in a lateral flow device that is cheap, fast, and can be used outside of the diagnostic lab. This RPA assay and lateral flow device provides a rapid, sensitive, and specific diagnostic test capable of providing surveillance and early diagnosis of CyHV-3 in the laboratory and field.

Introduction

Cyprinid herpes virus 3 (CyHV-3), also known as koi herpesvirus (KHV), is a highly pathogenic and contagious member of *Alloherpesviridae* (Pikarsky et al. 2004, Waltzek et al. 2009). Other closely related members of the group include carp pox herpesvirus (CyHV-1) and goldfish hematopoietic necrosis virus (CyHV-2). Initial reports describing the virus originated from Israel, the United States and Germany; however, CyHV-3 has since been reported worldwide (Hedrick et al. 2000, Choi et al. 2004, Grimmett et al. 2006, Garver et al. 2010, Taylor et al. 2010, Avarre et al. 2012, Cheng et al. 2011, Dong et al. 2013, Marek et al. 2010, Bondad-Reantaso et al. 2005, Calle, McNamara, and Kress 1999, Bretzinger et al. 1999).

Productive infection occurs in several tissues/organs, including but not limited to gills, eyes, skin, kidney, heart, and brain (Gilad et al. 2004, Miyazaki et al. 2008). Based on the severe clinical manifestations of disease, mortality rates have been reported to be 80-100% (Bergmann, Riechardt, et al. 2010, Dixon et al. 2009, Hedrick et al. 2000). As is consistent with other known herpesviruses, CyHV-3 also becomes latent in koi that recover from an initial viral infection (Eide et al. 2011, St-Hilaire et al. 2009). Latency is a hallmark of herpesvirus infection, and is characterized by the persistence of the viral genome within the host cells and the lack of virus particle production. The main site for latency of CyHV-3 has been identified to be white blood cells (WBC), specifically the B lymphocyte (Eide et al. 2011, Reed et al. 2014). Fish with latent CyHV-3 infection can live normally; however, they can shed infectious viruses and succumb to viral disease when they experience latency reactivation under stressful conditions. While the severity and consequences of disease have been devastating to the carp aquaculture industry as well as the ornamental koi trade, latency and reactivation highlight additional risk for subsequent reinfection of surviving populations and the spread to naïve groups of fish (St-Hilaire et al. 2005). Diagnostic methods to detect CyHV-3 must be able to detect the latent infection so that naïve populations are not unknowingly exposed to carriers, which would put them at risk of exposure to this deadly virus. Currently, no test is available that can detect latent infection. CyHV-3 becomes latent in less than 1% of cells in the peripheral white blood cells (Reed et al. 2015). Only a few copies of viral genome are present in the latently infected cells. Currently available diagnostic tests, such as enzyme-linked immunosorbent assay (ELISA) and PCR, often misdiagnose koi that are CyHV-3 latently infected. New tests that are capable of detecting low copy numbers of CyHV-3 genome are needed to diagnose CyHV-3 carriers and therefore prevent the spread of the virus.

Several diagnostic methods have been employed to detect CyHV-3, including ELISA (St-Hilaire et al. 2009), immunohistochemistry based assays (Aoki et al. 2011, Tu et al. 2014), hybridization assays (Monaghan et al. 2015, Saleh and El-Matbouli 2015), numerous PCR assays (Bercovier et al. 2005, Gilad et al. 2002, Ishioka et al. 2005, Bergmann, Riechardt, et al. 2010, El-Matbouli, Rucker, and Soliman 2007, Yuasa et al. 2012), and loop mediated isothermal amplification (Gunimaladevi et al. 2004, Soliman and El-Matbouli 2005, 2010, Yoshino et al. 2009). PCR-based strategies have been the “gold standard” as they are more sensitive than ELISA based methods and are less invasive than strategies requiring invasive tissue sampling, like many hybridization assays. However, these available assays all have limitations in specificity and sensitivity. Therefore, more sensitive, rapid and simple detection assays are needed for clinicians for use in field diagnostics.

Lateral flow (immuno) assay/ immunochromatography is a technique with broad spectrum applications including the detection of nucleic acids (Posthuma-Trumpie, Korf, and van Amerongen 2009). Nucleic acid detection is through primer sets with two different tags recognized in the lateral flow device by antibodies specific to the tags. As the sample is applied to a strip containing a polymeric material, it reacts with areas where specific antibody molecules have been affirmed to the strip resulting in a colorimetric signal. Each lateral flow device (LFD) has at least two lines, a test line where the sample is recognized and a control line to ensure the proper flow of sample through the strip (Posthuma-Trumpie, Korf, and van Amerongen 2009). In this study, a commercially available LFD detects nucleic acid in less than 10 minutes and has a simple and straightforward protocol that can be easily used without extensive technical training.

Recombinase polymerase amplification (RPA) is an emerging method for the isothermal amplification of nucleic acid (Piepenburg et al. 2006), and has

been employed for the detection of various pathogens (Abd El Wahed, El-Deeb, et al. 2013, Abd El Wahed, Patel, et al. 2013, Ahmed, van der Linden, and Hartskeerl 2014, Amer et al. 2013, Boyle et al. 2013, Boyle et al. 2014, del Río et al. 2014, Euler, Wang, Otto, et al. 2012, Euler, Wang, Nentwich, et al. 2012, Jaroenram and Owens 2014, Krõlov et al. 2014, Mekuria, Zhang, and Eastwell 2014, Tsaloglou et al. 2015, Xia et al. 2014, Xia et al. 2015, Zaghloul and El-Shahat 2014, Zhang et al. 2014). The RPA method utilizes three main enzymes to amplify template DNA: recombinases, single stranded binding protein (SSB), and a strand-displacing polymerase (Piepenburg et al. 2006). Exponential amplification of target amplicons proceeds by the use of two opposing oligonucleotide primers similar to PCR. The length of the RPA primers, >30 bp, is longer than those primers commonly used in traditional PCR; melting temperature differences are inconsequential because thermocycling is unnecessary for the RPA reaction to proceed. Amplification in a RPA reaction starts by primers forming a complex with recombinase enzymes that pair primers to their homologous template sequences (Piepenburg et al. 2006). SSB then bind to displaced template DNA strands and synthesis proceeds with a strand displacing polymerase (Piepenburg et al. 2006). RPA has been used as a rapid and sensitive method for amplifying nucleic acid of pathogens. Here, we describe the application of RPA to detect CyHV-3 genome in latently infected koi and directly compare results to a PCR-based assay.

In this study, we employed RPA to isothermally amplify CyHV-3 DNA from latently infected koi fish and detected the product using a lateral flow device.

Materials and Methods

Koi sampling

Thirteen adult koi fish with confirmed exposure to CyHV-3 were used in this study with no clinical signs of CyHV-3 infection within the past two years. Koi were maintained at the Oregon State University John L. Fryer Salmon Disease Laboratory (OSU-SDL) and blood samples were collected as previously described (Reed et al. 2014). White blood cells (WBC) were isolated by a Ficoll-Paque PLUS gradient according to the manufacturer's instructions (GE Healthcare, United Kingdom).

Template preparations

The genomic DNA purified from strain CyHV-3 (KHV-U) was used a positive control in this study. CyHV-3 genomic DNA was diluted to 100, 50, and 10 copies per sample estimated by spectrophotometric assessment by NanoDrop. A minimum of seven replicates was tested for each dilution. Total DNA was extracted from koi white blood cells (WBC) with the High Pure PCR template preparation kit (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions; DNA was eluted in nanopurified water. 0.5 µl of total extracted DNA (500 ng-1 mg) was used in the RPA assay as template.

To investigate the specificity of the RPA assay samples known to be negative for CyHV-3 were used as control. Total DNA was extracted from a closely related cyprinid species, the goldfish (*Carrasius auratus*) which included WBC and skin samples. Additionally, a negative control sample of total DNA from a goldfish with confirmed Cyprinid herpesvirus 2 (CyHV-2) infection was also used as negative control.

Primer design

Primers suitable to RPA amplification were designed based on the CyHV-3 reference sequence (NCBI accession no. NC_009127.1). RPA primers were designed to detect a 230 bp region of the major capsid protein of CyHV-3 (Table 5.1). The primers for quantitative TaqMan PCR were selected as

previously described (Table 5.1)(Gilad et al. 2004). For product detection on a lateral flow device, a 5' modification of either FAM or biotin was added to each original RPA primer.

Quantitative PCR

To detect and quantify CyHV-3 genome from each koi, ~500 ng of total DNA extracted from WBC was used as template in quantitative PCR with primers KHV-86f and KHV-163r and TaqMan probe KHV-109p as previously described (Reed et al. 2014, Gilad et al. 2004).

RPA assay

To detect CyHV-3 genome in koi WBC using RPA, the TwistAmp Basic kit (TwistDx, LTd., Cambridge, United Kingdom) with the RPA specific primers (Table 5.1) were utilized. The RPA assay contained a lyophilized pellet premeasured and distributed by the manufacturer that was dissolved using a mix of 29.5 µl rehydration buffer, 0.5 µl DNA template, 3 µl primers (300 nM each) and 14.5 µl water to a final reaction volume of 50 µl. Magnesium acetate (2.5 µl, 280 nM) was used to initiate the RPA reaction as described in the manufacturer's instructions. A negative control (water with no DNA) sample was included in each run along with a positive control for assay runs involving koi WBC DNA samples, which were run in duplicate. The reaction was conducted at 37°C (unless otherwise specified) on a heat block for 20 minutes and purified using a GeneJET DNA purification kit (Thermo Scientific, Waltham, MA) for samples observed by gel electrophoresis.

To confirm the correct DNA product was amplified, Sanger sequencing was performed at the Center for Genome Research and Biocomputing (CGRB) at Oregon State University. Following gel electrophoresis, selected bands of the approximated product size were visualized on an LED blue light transilluminator and were excised using a razor blade. DNA was purified

using a GeneJet kit eluted using diH_2O. Sequencing results were aligned to the predicted CyHV-3 sequence by pairwise alignment tool.

Amplification products were detected by either visualization by gel electrophoresis on a 1.5% agarose gel stained with SYBR Safe (Life Technologies, Carlsbad, CA) following DNA purification or by lateral flow strip detection (LFD).

Lateral Flow Device application

For rapid detection of CyHV-3 genome, RPA product was visualized using a lateral flow device. Briefly, 5' modified primers (Table 5.1) were substituted for original RPA primers in the RPA assay, and end products now labeled with both FAM and biotin were diluted with supplied buffer and the diluted product was applied onto the PCRD-2 strips (Forsite Diagnostics, United Kingdom). Results were assessed by visual detection of colorimetric signal on the lateral flow strip which detects the combination of the 5' primer modifications, FAM and biotin, by anti-FAM and anti-biotin antibodies that are immobilized together at the test line (T-line). A control line (C-line) is incorporated as a flow check control. Negative results are indicated on the strip by a single line at the C-line position and no color detected on the T-line. Positive results are indicated by lines at both the C- and T-line positions. Strips that do not develop a line on the C-line are discarded as faulty.

Results

Detection of CyHV-3 using the RPA assay

To test the ability of the RPA assay to detect CyHV-3 DNA, DNA purified from the KHV-U strain at 100, 50, and 10 copies was used as template in the RPA assay. As shown in Figure 5.1, an approximate ~230 bp product was detected in reaction with templates at 100, 50, and 10 copies of CyHV-3 genomic DNA (Figure 5.1, lanes 3, 4, and 5 respectively). No band was seen from reactions

without CyHV-3 DNA (nanopure water) when ran in parallel to CyHV-3 DNA samples (Figure 5.1 lane 1, Table 5.2). This result was consistent with all replicates for each of the dilutions tested. To confirm the amplification is specific for the CyHV-3 genome, RPA products were examined by Sanger sequencing. As shown in Figure 5.2, all the RPA products show high sequence identity to the CyHV-3 reference DNA. To test the RPA specificity, templates from CyHV-2 and total DNA of goldfish (*Carrasius auratus*), which is known to be CyHV-3 free, were also evaluated (Figure 5.1, Table 5.2). As shown in Figure 5.1, lanes 7-8, no product was amplified from any of the negative control samples, which suggests the primers selected for CyHV-3 is specific for the virus. Our results demonstrated that CyHV-3 RPA is specific and capable of detecting viral genome copy number as low as 10 copies.

Evaluation of temperature effects on RPA amplification of CyHV-3

To investigate the range of temperatures in which the RPA assay can be performed, positive control DNA was diluted to 10 copies per reaction and the RPA assay was run at temperatures of 41°C, 39°C, 35°C, 33°C, as well as at the manufacturer's recommendation standard of 37°C . As seen in Figure 5.4, RPA amplification of CyHV-3 DNA was confirmed across the temperature range of 33-41°C. While positive amplification was shown at each temperature tested, efficiency of the reaction was variable as seen by the signal strength of each band.

Detection of CyHV-3 by RPA assay in latently infected koi

To determine the ability of the RPA assay to detect latent CyHV-3 infection in koi, total DNA of WBC from latently infected koi was evaluated by the RPA assay. As shown in Figure 5.3, CyHV-3 DNA was amplified from 12 of the 13 samples by the RPA assay. All fish samples were tested by the RPA assay with a minimum of two replicates using total DNA (500ng-1mg) in each

reaction. The same samples of DNA from each of the13 fish were also analyzed by quantitative TaqMan PCR, where only one sample was tested positive. Amplified product from three samples were also selected for further confirmation of RPA results by Sanger sequencing. As shown in Figure 5.2, all RPA products from three different koi have DNA sequence with 100 % identity to the CyHV-3 genome (NCBI accession no. NC_009127.1). This result demonstrated that the RPA assay is capable of detecting CyHV-3 DNA in latently infected fish and has a greater sensitivity than the quantitative PCR as described previously (Gilad et al. 2002).

Detection of CyHV-3 RPA products on a Lateral Flow Device

To test the application of this CyHV-3 RPA assay as a rapid detection assay, RPA products were applied to a lateral flow device. The RPA products from six koi WBC DNA samples were added to a LFD containing PCRD-2 strips as well as negative control (RPA product amplified with no DNA template) and positive control (RPA product amplified from 50 copies KHV-U DNA) products. As seen in Figure 5.5, a positive colorimetric signal was detected after RPA from the positive control sample and from the WBC of three representative latently infected koi but not from the negative control sample. All six latently infected koi samples confirmed positive by gel electrophoresis also produced a positive signal by LFD. This data demonstrates detection of purified RPA products by LFD as an alternative to visualization by gel electrophoresis.

Discussion

The need for a highly sensitive assay capable of detecting latent infections is paramount as undiagnosed latent fish could be introduced to naïve populations leading to subsequent infection, loss of fish, and monetary losses. The RPA assay we report herein is a rapid and robust alternative test

for detecting CyHV-3. This RPA assay is specific to CyHV-3 and is capable of detecting as little as10 copies of CyHV-3 genome (Figure 5.1, Table 5.2). The RPA assay consistently and repeatedly amplified CyHV-3 genome, while negative control reactions remained unamplified. The assay successfully detected CyHV-3 DNA across the temperature range of 33-41°C, suggesting that the RPA assay can be performed at any one temperature without a thermocycler (Figure 5.4). Using the RPA assay, CyHV-3 was detected in all previously confirmed latently infected fish, and provided a more sensitive detection of latently infected koi as directly compared to quantitative PCR (Figure 5.3). In addition, we illustrated the ability of the end products to be visualized by LFD detection (Figure 5.5). The data we present demonstrated the advantage and resilience of using RPA in conjunction with LFD to detect CyHV-3.

Only one fish produced a negative result by this RPA assay. This fish was exposed to CyHV-3, as it was housed in a tank with latently infected koi, but has never exhibited active infection. Additionally, to date, this fish has not been confirmed by any other method as having latent infection. In explanation, it is possible this exposed fish could have only a few cells latently infected thereby the genome copy number is below the detection limits. It is also possible very few latently infected cells were collected during sampling. While the 12 confirmed latently infected fish used in this study had previously been positively identified by PCR strategies, in this study, only one was identified as positive by detection with quantitative PCR. This can be explained in several ways as the quality of DNA extraction could have varied from previous samples tested or the circulating genomic copies may fluctuate over time. However, the direct comparison of RPA with quantitative PCR demonstrates that RPA is a much more sensitive assay for detecting latent CyHV-3 infections.

RPA is an isothermal method of DNA amplification; therefore, it can be performed at one constant temperature and expensive machinery necessary to perform thermocyling is not required. RPA produces reliable results at a wide range of temperatures, further demonstrating its robust nature and highlighting its flexibility over traditional PCR strategies. The optimum reaction temperature of the RPA enzymes is near 37°C and although our assay is successful at a range of temperatures, running at 37°C will increase the chance of optimum enzyme performance. Ideally a diagnostic assay could be performed by minimally trained technicians in a patient-side manner and produce results as quickly as possible to prevent translocation of potentially infected fish and subsequent transmission of the disease.

We have shown that LFD technology can be used in conjunction with our RPA assay demonstrating the potential development of RPA as a field assay. Detection by LFD adds less than 10 minutes to the already rapid assay time and omits the use of expensive quantitative machinery associated with real time detection strategies and gel electrophoresis equipment otherwise necessary to visualize the amplified product. As an alternative to lateral flow device, the dynamic nature of the RPA assay allows for the addition of a fluorescent probe to detect amplified results in real time. The speed, sensitivity, and overall dynamic nature of the RPA assay highlight a viable option for future development of a CyHV-3 specific rapid diagnostic test capable of detection in an onsite manner. In future developments we would like to improve upon the handling and DNA extraction process for a more accessible protocol which would decrease detection time and further eliminate technical equipment needed to adequately employ this assay in the field.

RPA detection of CyHV-3 has many advantages when compared to other detection methods. An ELISA based detection assay has been reported to be

less sensitive than TaqMan PCR (Eide et al. 2011)); in a direct comparison, this RPA assay is more sensitive than quantitative PCR assay as latent CyHV-3 was detected in more fish using RPA than qPCR. In addition to sensitivity, RPA compared to other genomic detection strategies has additional advantages. RPA is faster (amplification in about 20 minutes), and ease of performance is equal or greater than PCR, with less expensive and sophisticated equipment required. Fluctuations in temperature have little effect on the results and a wide range of temperatures provides flexibility and will lead to more successful application of the diagnostic test. RPA has true promise for further development of a rapid, affordable, easy, and sensitive diagnostic test to detect CyHV-3.

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Table 5.1. RPA and quantitative Taqman PCR primers used for the detection of CyHV-3 genome.

Name	Sequence (5'-3')	5' modification[a]
	RPA	
KHV-MCP2F	TTCTTCAAGCCGGACGCCTTCAACGTGCAGCG	FAM
KHV-MCP2R	TTCTCCAGGCGGCTCATGACGCTGGTGTTCTCGG	Biotin
	Quantitative PCR[b]	
KHV-86f	GACGCCGGAGACCTTGTG	
KHV-163r	CGGGTTCTTATTTTTGTCCTTGTT	
KHV-109p	CTTCCTCTGCTCGGCGAGCACG	

[a] Modified primers were added for product detection via LFD.

[b] (Gilad et al. 2004).

Table 5.2. Sensitivity and Specificity of RPA assay for the detection of CyHV-3.

Sample	Detection of CyHV3 DNA by RPA assay
CyHV-3 genome: 100 copies	Positive
CyHV-3 genome: 50 copies	Positive
CyHV-3 genome: 10 copies	Positive
Water (no DNA)	Negative
Goldfish skin	Negative
Goldfish WBC	Negative
CyHV-2 positive goldfish WBC	Negative

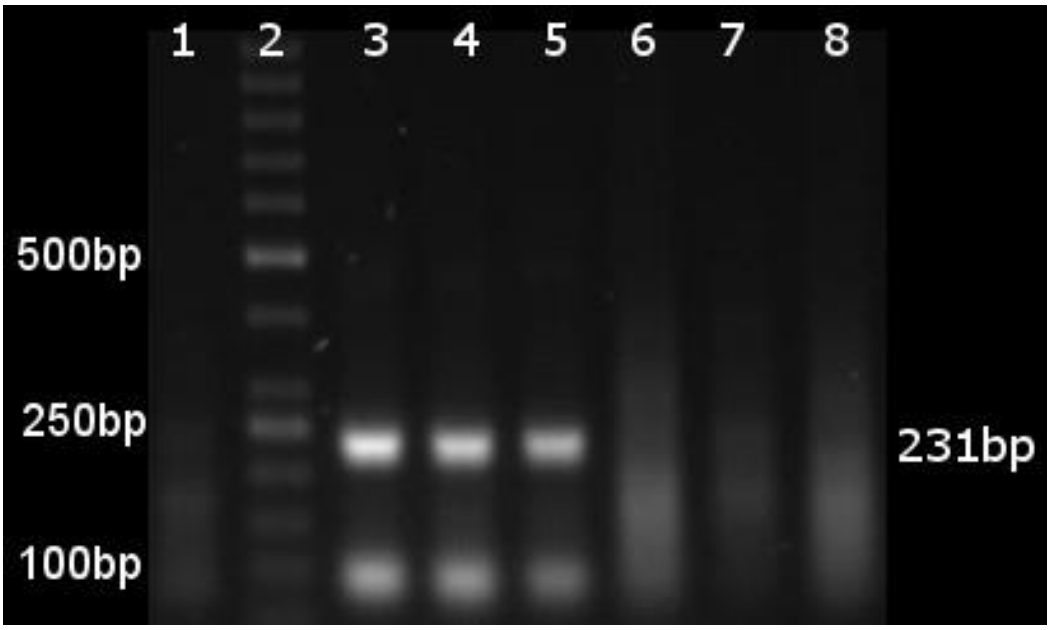

Figure 5.1. Electrophoresis of RPA amplification products following DNA purification. Products were run at 85 volts for 70 minutes in 1.5% of agarose in TAE buffer. The numbers in the left margin indicate the positions in base pairs for selected bands of the DNA marker (Thermo Scientific™ GeneRuler™ 50 bp DNA Ladder, 50-1000 bp).The number in the right margin indicates the predicted 230 bp size of RPA amplification. Lane 1 is the negative control (water as template), lane 2 is a 50 bp DNA ladder. Lanes 3-5 are RPA products from 100, 50, and 10 copies of CyHV-3 genomes respectively as template. Lane 6 is RPA product from a goldfish skin DNA, lane 7 is RPA product from goldfish WBC DNA, and lane 8 is RPA product using WBC DNA from a fish positive for Cyprinid herpesvirus 2.

```
CyHV-3_50       ------------------------------------------------------------AC 2
CyHV-3_10       ------------------------------------------------------------AAC 3
Koi_1           ------------------------------------------------------------AAC 3
Koi_2           ------------------------------------------------------------GAAC 4
NC_009127.1     TTCTTCAAGCCGGACGCCTTCAACGTGCAGCGCTTCTACACCACCTGCCCCTTCCAGAAC 60
Koi_3           ------------------------------------------------------------GAAC 4
                                                                             **

CyHV-3_50       CCCTACCTGAACGAGATGCAGGCCGCGCTGCACAGGATCCTCGGCCAGCTCGGCGATCCC 62
CyHV-3_10       CCCTACCTGAACGAGATGCAGGCCGCGCTGCACAGGATCCTCGGCCAGCTCGGCGATCCC 63
Koi_1           CCCTACCTGAACGAGATGCAGGCCGCGCTGCACAGGATCCTCGGCCAGCTCGGCGATCCC 63
Koi_2           CCCTACCTGAACGAGATGCAGGCCGCGCTGCACAGGATCCTCGGCCAGCTCGGCGATCCC 64
NC_009127.1     CCCTACCTGAACGAGATGCAGGCCGCGCTGCACAGGATCCTCGGCCAGCTCGGCGATCCC 120
Koi_3           CCCTACCTGAACGAGATGCAGGCCGCGCTGCACAGGATCCTCGGCCAGCTCGGCGATCCC 64
                ************************************************************

CyHV-3_50       AACGCAGACATGTTCGCGCCGGGCAAGCGCAACGCCGTGCTCACCACCACCATCAAGCAC 122
CyHV-3_10       AACGCAGACATGTTCGCGCCGGGCAAGCGCAACGCCGTGCTCACCACCACCATCAAGCAC 123
Koi_1           AACGCAGACATGTTCGCGCCGGGCAAGCGCAACGCCGTGCTCACCACCACCATCAAGCAC 123
Koi_2           AACGCAGACATGTTCGCGCCGGGCAAGCGCAACGCCGTGCTCACCACCACCATCAAGCAC 124
NC_009127.1     AACGCAGACATGTTCGCGCCGGGCAAGCGCAACGCCGTGCTCACCACCACCATCAAGCAC 180
Koi_3           AACGCAGACATGTTCGCGCCGGGCAAGCGCAACGCCGTGCTCACCACCACCATCAAGCAC 124
                ************************************************************

CyHV-3_50       TACTACCAGTTCCTCACCGAGAACACCAGCGTC----------------- 155
CyHV-3_10       TACTACCAGTTCCTCACCGAGAACACCAGCGTC----------------- 156
Koi_1           TACTACCAGTTCCTCACCGAGAACACCAGCGTC----------------- 156
Koi_2           TACTACCAGTTCCTCACCGAGAACACCAGCGTC----------------- 157
NC_009127.1     TACTACCAGTTCCTCACCGAGAACACCAGCGTCATGAGCCGCCTGGAGAA 230
Koi_3           TACTACCAGTNCCTCACCGAGAACACCAGCGTC----------------- 157
                ********** **********************
```

Figure 5.2. Sequence alignment of RPA products to CyHV-3 genome. The sequences were aligned to CyHV-3 reference sequence (NCBI accession no. NC_009127.1). Sequences of three RPA products from koi WBC samples are named Koi_2, Koi_1 and Koi_3. Sequences of RPA products from 50 and 10 copies of CyHV-3 and are named CyHV-3_50 and CyHV-3_10 respectively. The number to the right of each row set indicates the nucleotide location in reference to the 230bp Predicted sequence. Sequences were aligned using the CLUSTAL 2.1 multiple sequence alignment tool.

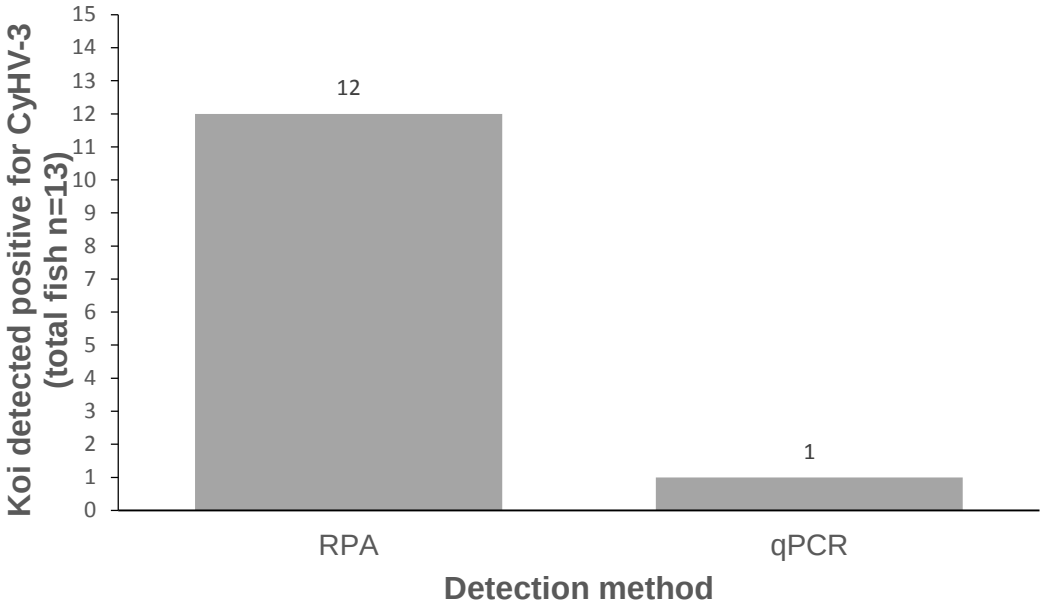

Figure 5.3. Summary of positive CyHV-3 detection from latently infected koi. Detection of DNA extracted from WBC samples (n=13) from exposed or latently infected koi using RPA and quantitative PCR (qPCR).

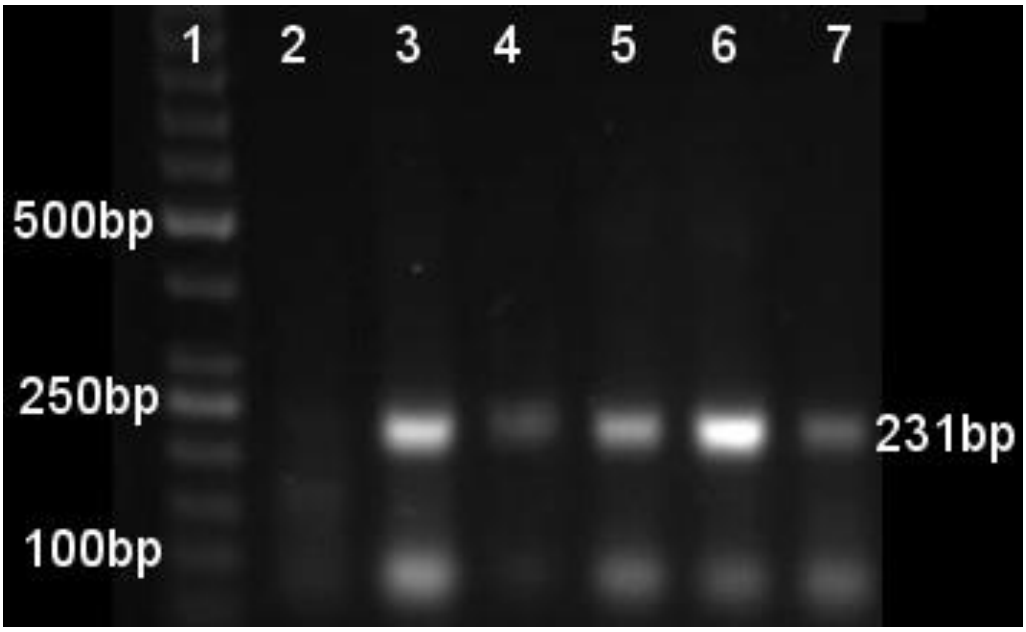

Figure 5.4. Reaction temperature range of RPA amplification of CyHV-3. The numbers in the left margin indicate selected bands of the DNA marker, and the estimated amplified product size is indicated at 230 bp in the right margin. Lane 1 is a 50bp DNA ladder **(**Thermo Scientific™ GeneRuler™ 50 bp DNA Ladder, 50-1000 bp**)**, lane 2 is the negative control (water as template). Lanes 3-7 represent RPA amplification products from 10 copies of CyHV-3 genome per reaction at temperatures 41°C, 39°C, 37°C, 35°C, and 33°C respectively.

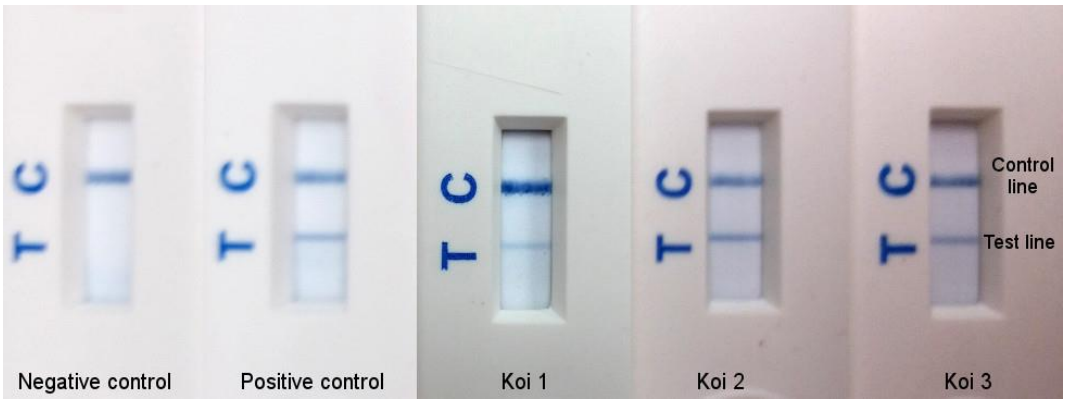

Figure 5.5. Detection of CyHV-3 product from RPA assay in lateral flow detection strips. The control line is labeled as C which indicates the strip is working as predicted. The test line is labeled as T which indicates the presence of doubly–labeled (with FAM and biotin) amplified product. Samples from left to right: Negative control: water as template, positive control: 50 copies of KHV-U, Koi 1-3: three representative products amplified from total DNA of WBC DNA from latently infected koi.

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Chapter 6. Concluding remarks

Viral disease has an immeasurable impact on the human population and is incredibly important to both human health and our economies. Whether it is the health of our own persons, our pets, our food supply, or the organisms we share the world with, viral disease plays a major role in the burden of disease in every organism that humans interact with. There are many important avenues of research for viral disease left to explore, including those for host-viral pathogen interactions and diagnostics. Both of these topics continue to be areas of interest and are in great need as new viral diseases emerge. The knowledge gained by investigating host-viral interactions aids in our overall understanding of viral disease and disease pathogenesis and can be applied to a wide range of applications including: treatment, prevention, and even diagnostics. Diagnostic strategies are ever evolving, as faster, cheaper, easier, and more reliable methodologies are desired.

The goal of these studies was twofold. First, to investigate and utilize host-viral pathogen interactions in respiratory RNA viruses for better understanding of viral pathogenesis within a host system, and to exploit the interaction between host and virus for our benefit. Secondly, to apply new methodology for diagnostics that improve upon current detection strategies, focusing on issues of these diagnostics and developing a better assay to detect viral disease.

In Chapter 2, we apply knowledge of host-viral pathogen interactions to the influenza vaccine production challenge. Vaccines are undeniably essential for prevention of disease burden and their manufacture is a multi-billion dollar industry worldwide. The influenza vaccine is unique as the vaccine composition changes yearly. Despite the licensing of alternative methods of production, including cell culture methods, the antiquated, egg-based manufacture of influenza still predominates. While many factors most likely contribute to this phenomenon, it is clear that new methodologies that

improve cell culture-based influenza vaccines are needed to advance the current technologies in order to make them more attractive to finally move away from the egg-based system. This process is certainly beginning but continued efforts are also desirable. We demonstrated that by treating cells with a chicken IFNα specific PPMO we could increase viral replication and propagate more virus in chicken cells. Treatment of our PPMO was not toxic to the cells and the design of the PPMO allows for self-directed delivery requiring no additional manipulations. This technology is advantageous to commonly used RNA interference platforms for expression modification due to several factors: PPMOs have self-directed delivery, they generally have low toxicity, and are more stable and do not suffer from nonspecific activity commonly seen by using siRNA RNA interference. Our work using PPMOs to increase viral replication in cell culture demonstrates the usefulness of PPMOs technology as applied to vaccine production. Permanent deletion of genes, while possible, would be time consuming and expensive. PPMOs could be used as a quick, easy, and cheap tool to screen candidate genes before permanent deletion is attempted. We have evaluated how the knockdown of IFNα affects the production of influenza virus in cell culture and have shown that it is a target for suppression of its protein production that results in enhanced virus yields for vaccine purposes. It would be advantageous to evaluate additional host genes for protein suppression via PPMO treatment to see if other targets would similarly or superiorly increase viral replication. It would also be valuable to assess how targeting multiple host genes at once would affect the growth of influenza in cell culture. As most vaccines are made in the egg system, it might also be useful to find gene targets for PPMO treatment that would work in the egg. We were unable to modulate expression in the egg system using the PPMO to target IFNα, but this is a potential avenue for future research that may have promising application.

In Chapter 3, we were looking to further understand RSV pathogenesis and the host-viral pathogen interaction between RSV and the host gene Mcl-1. We sought to characterize how Mcl-1 functions in relation to RSV infection by using a murine cell line with a Mcl-1 knockout. Our work was quite surprising, as the knockout cells caused significant increase of viral replication and phenotypic changes within infected cells; this increase in viral titers for knockout cells was also recorded for influenza virus. These results indicate that the upregulation of Mcl-1 associated with RSV infection is most likely an antiviral strategy of the host, not a viral induced mechanism to increase cell viability for viral propagation. Apoptosis modulation has been studied and shown to either be a pro or antiviral strategy dependent on the portion of the pathway studied and the viral pathogen examined. The work described herein is the first time Mcl-1 has been specifically examined in direct context of host-viral pathogen interactions. Our data also indicated another surprising result in our experiments involving apoptosis in these cells. Mcl-1 has been shown to be upregulated in RSV infection as early as 2 hours after initial infection. As Mcl-1's chief function is anti-apoptotic, it would make sense to see apoptosis being induced early in the infectious cycle, however, we did not see this suggesting Mcl-1 may have a role as host induced anti-viral response. While we could induce increased viral replication in WT cells by treatment of an apoptosis inducing compound, treatment of knockout cells with an apoptosis inhibiting compound had no effect on viral titers. Our apoptosis results and the confocal micrographs depicting morphological changes in syncytial formation implicate that apoptosis regulation by Mcl-1 may not be the sole function of Mcl-1 as indicated in early induction during RSV replication and increased RSV replication in Mcl-1 knockout cells. Further work into the mechanism that is driving this observation is needed, but has the potential to display a novel role of Mcl-1 in viral infection. It would also be interesting to

investigate how Mcl-1 suppression would affect other classes of viral pathogens.

In Chapters 4 and 5, we developed a rapid diagnostic assay based on the RPA method to detect viral genome for two important viral pathogens: DENV and CyHV-3. RPA is an isothermal method to amplify DNA that is attractive for diagnostic strategies of the future. PCR has been shown in previous work to be capable of high sensitivity and specificity that you would typically associate with PCR based techniques while being faster and cheaper in both machinery and resource consumption. RPA has also been evaluated in several studies for its use as a point-of-care based diagnostic, useful in resource-limited settings. RPA is robust and adaptable; the assay can handle the input of a wide range of template concentrations, reaction temperatures, storage temperatures, and several detection platforms can be utilized dependent on the needs of those using the diagnostics. In addition, while the reaction is isothermal, the temperature of the reaction does not require a stable and consistent temperature, valuable in resource limited settings where stable temperatures may be difficult to maintain. Like PCR, RPA requires primers to amplify, a slight drawback as the reliability of the assay depends on how effective the primers are and whether suitable primers can be designed. However, RPA primers are not reliant on melting temperature making it much easier to find appropriate primer sets. Another drawback addressed in investigations of RPA, as a point-of-care diagnostic is the preparation of template needed for RPA amplification. DNA or RNA must be extracted to serve as a template for DNA amplification. Further work is needed to simplify the extraction procedure, and would be a major breakthrough to making RPA an elite method for not only viral but all other pathogen diagnostics.

In Chapter 4, we assessed the suitability of RPA to detect dengue virus type 2. We show that RPA was very sensitive and specific in all the experiments to detect dengue genome from both laboratory spiked and clinical samples. Further testing is essential to confirm the reliability of our DENV2 specific RPA assay and we are working to develop RPA primers for each of the additional DENV serotypes. Multiplexing the reactions is work for the future that would make serotyping with RPA possible in a point-of-care setting, whether it be by developing a LFD that can capture and distinguish between serotype specific amplification products or through real-time multiplexing through different serotype specific fluorescent probes.

In Chapter 5, we applied RPA to detect CyHV-3. While the virus is a veterinary relevant fish pathogen, infections have a tremendous economic impact to humans. Worldwide, carp is a leading fish for human consumption and the ornamental koi trade has also suffered from loss due to CyHV-3 infections. A major issue in the detection of CyHV-3 is that latently infected fish are hard to diagnose. By applying RPA as a diagnostic assay capable of detecting latently infected fish we have demonstrated an improved diagnostic strategy that has the potential to result in real world application with economic impact.

The studies within this dissertation have led to further understanding of viral disease and the potential for further research and collaborations with other investigators in both the academic institutions and industry.

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Appendices

Appendix 1: Abstracts of Additional Publications

Appendix 1.1 Identification of Unique Blood and Urine Biomarkers in Influenza Virus and Staphylococcus aureus Co-infection: A Preliminary Study.

Authors: **Prescott MA**, Pastey MK.

Journal: Biomarker Insights December 5, 2010

Abstract

Each year, there are estimated to be approximately 200,000 hospitalizations and 36,000 deaths due to influenza in the United States. Reports have indicated that most deaths are not directly due to influenza virus, but to secondary bacterial pneumonia, predominantly staphylococcal in origin. Here we identify the presence of candidate blood and urine biomarkers in mice with *Staphyococcus aureus* and influenza virus co-infection. In this pilot study, mice were grouped into four treatments: co-infected with influenza virus and S. aureus, singly infected with influenza virus or S. aureus, and a control group of uninfected mice (PBS treated). Gene expression changes were identified by DNA-microarrays from blood samples taken at day five post infection. Proteomic changes were obtained from urine samples collected at three and five days post infection using 2-D DIGE followed by protein ID by mass spectrometry. Differentially expressed genes and/or proteins were identified as candidate biomarkers for future validation in larger studies.

Appendix 1.2 Basant, a Polyherbal Topical Microbicide Candidate Inhibits different Clades of Both CCR5 and CXCR4 Tropic, Lab-Adapted and Primary Isolates of Human Immunodeficiency Virus-1 in Vitro Infection

Authors: Maciej B Maselko, Rupali Joshi, **Meagan Prescott**, GP Talwar, Smita Kulkarni, and Manoj Pastey

Journal: Journal of Virology & Antiviral Research November 12, 2014

Abstract

Topical microbicides applied to the vaginal mucosa as pre-exposure prophylactic measures for controlling sexually transmitted diseases have great potential to combat theHIV-1 pandemic in the developing world. Basant is a polyherbal topical microbicide candidate with demonstrated activity against CXCR4 co-receptor tropic HIV-1 as well as fungal and bacterial sexually transmitted infections. The current study demonstrates that Basant is effective against both CCR5 and CXCR4 tropic HIV-1 lab-adapted strains and primary isolates from different clades. We also investigate the kinetics of Basant inhibition as compared to the fusion inhibitor Enfuvirtide and report that Basant inhibits HIV-1 at an early stage of infection. We conclude that Basant may be considered as a potential candidate for use as an effective vaginal microbicide in preventing HIV-1 transmission.