ANALYSIS OF THE SUPPRESSIVE EFFECTS BY REOVIRUS ON ROTAVIRUS REPLICATION DURING CO-INFECTIONS

by

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June 22, 2010

ABSTRACT

ANALYSIS OF THE SUPPRESSIVE EFFECTS BY REOVIRUS ON ROTAVIRUS REPLICATION DURING CO-INFECTIONS

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Reovirus and rotavirus are both members of the Reoviridae family and commonly occur in children. Rotavirus induced diarrhea is one of the leading causes of death in children under the age of five worldwide. Identifying new treatment options for an unnecessarily deadly diarrheal infection caused by rotavirus would benefit hundreds of thousands of children a year. Currently, only oral rehydration therapy or costly vaccinations are available to treat or prevent rotavirus diarrhea. Even with their prevalence, little is known about how these two viruses (reovirus and rotavirus) interact when co-infecting cells. Determining these interactions could lead to new treatment options for rotavirus infections. Using the three known human reovirus serotypes (MRV-1LA, MRV-2JO, and MRV-3DE) *in vitro* co-infections were performed with Rhesus rotavirus (RRV) in MA-104 cells. These infections revealed that infectious RRV particle production was greatly inhibited by the presence of MRV-1LA and MRV-2JO, but not affected by the presence of MRV-3DE. To determine which reovirus genes were responsible for the inhibition, co-infections using RRV and two sets of mammalian orthoreovirus monoreassortants (MRV-1LA with MRV-3DE genes and MRV-3DE with MRV-2JO genes) were performed in MA-104 cells. It was determined that when the S2 and S3 genes of MRV-1LA and

MRV-2JO were present in the co-infections, infectious particle production was completely inhibited. Next, *in vivo* studies were performed using Balb/c mice to determine if co-infections with RRV and MRV-1LA, MRV-2JO, or MRV-3DE resulted in lessened diarrhea in comparison to mice singularly infected with RRV. It was found that mice simultaneously co-infected with RRV and MRV-1LA developed diarrhea to a lesser degree than mice singularly infected with RRV. These results indicate that MRV-1LA has the ability to inhibit rotavirus diarrhea in mammals when co-infection occurs. This inhibitory property of MRV-1LA could lead to potential treatment options of rotavirus infections and help many children every year.

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CHAPTER 1

INTRODUCTION

Members of the Reoviridae family are common viruses found in the environment. Two members, Reovirus and Rotavirus, are ubiquitous among mammals. Almost all human children have developed antibodies to at least one serotype of each virus by the age of five. Reovirus normally results in asymptomatic or minor infections in most children and adults. Rotavirus, on the other hand, is commonly associated with illness in children.

Rotavirus is the most common cause of severe gastroenteritis in infants and children under the age of five in the world. It is estimated that rotavirus is responsible for approximately 500,000[1] deaths a year worldwide among children under the age of five.[2-4] Worldwide, by the age of five, one in five children require medical care for rotavirus induced gastroenteritis, one in 65 is hospitalized, and one in 293 dies.[5] Children from the poorest regions of the world account for 82% of all rotavirus deaths.[6]

To date, there are no cures for rotavirus infections. Two vaccines are currently available in the U.S. but are extremely expensive and require a good deal of cooperation between parents and doctors to adhere to their complicated and strict dosing regime. Their costs and dosing schedules make them a challenge to use worldwide, especially in underdeveloped regions. The WHO Strategic Advisory Committee on Immunization (SAGE) has issued a recommendation for inclusion of rotavirus vaccination of infants in all national immunization programs. Inter Press Service adds that "while public health officials praise the rotavirus vaccination's potential to combat child mortality and improve children's health in Africa and Mexico … the successful implementation of this recommendation presents a real and serious challenge in poor and middle-income countries." Such challenges include the storage and shipment of rotavirus vaccines, program implementation and vaccine cost.

Historically, it has been shown that co-infections of certain viruses *in vitro* and *in vivo* can result in decreased production of one of the involved viruses.[30,40-41] This hypothesis was tested to determine if co-infection of reovirus and rotavirus could result in lessened infectious rotavirus production.

CHAPTER 2

BACKGROUND

2.1 Reovirus Infections

Members of the Reoviridae family are widespread in the environment. Two members, Reovirus and Rotavirus, are omnipresent among mammals. Almost all human children have developed antibodies to both viruses well before the age of five. Reovirus (*R*espiratory *E*nteric *O*rphan virus) normally causes asymptomatic infections in most children and adults. Reovirus has been found in normal healthy individuals showing no signs of illness. It has also been found in individuals showing signs of illness such as respiratory symptoms, gastrointestinal symptoms, and/or paralysis, to list a few. Since it has been found in both healthy and ailing individuals it is often referred to as an "orphan virus", hence its name origin. It cannot definitively be designated as a cause of illness, so it is assumed to be a 'benign' virus in most individuals meaning that it causes asymptomatic infections.

Although reovirus is mostly asymptomatic it has been shown to result in symptomatic infections in some individuals. If symptoms are present in individuals, they can include fever, abdominal cramps, diarrhea, vomiting, respiratory illness, hepatitis, and central nervous system illness, including paralysis.[7] There are three serotypes of reovirus that are currently known to infect humans: Mammalian orthoreovirus 1 Lang (MRV-1La) MRV-1LA, Mammalian orthoreovirus 2D5/Jones (MRV-2Jo) MRV-2JO, and Mammalian orthoreovirus 3 Dearing (MRV-3De) MRV-3DE. MRV-1LA and MRV-2JO cause most cases of disease in infected individuals and are responsible for the most severe disease symptoms. MRV-2JO is the most neurotropic of the three serotypes followed by MRV-1LA then MRV-3DE.[8-16] MRV-3DE usually results in only minor, if any, disease associated with infection, however, it has been shown to cause jaundice in newborns.[17] MRV-3DE is usually so benign that it is currently being looked at as a

cancer treatment because it has shown oncolytic properties without negative side effects for the patient.[17]

2.1.1. Reovirus Vaccines

There are not currently any vaccines against the known reovirus serotypes because their infections are usually mild or asymptomatic. Infected individuals with symptoms usually recover quickly. Vaccine development against reovirus infections would be of no real value. As a matter of fact, reovirus vaccines against cancer are the current trend with reovirus. Reolysin® is the formulation of human reovirus currently in Phase II trials against various types of cancer in the U.S., Canada, and Europe and it is showing some positive results. Reovirus replicates in cells containing the active *Ras* pathway, which is common in many tumor cells. This replication causes the lysis of tumor cells. It has also been found that tumor antigens that are generated by this viral oncolysis may actually educate the immune system to recognize and kill tumor cells on its own.

2.2 Rotavirus Infections

Rotavirus, unlike Reovirus, is most commonly associated with illness in children. Most healthy adults are immune to rotaviral infection because exposure during childhood usually results in lifelong active immunity.[3] Rotavirus is the most common cause of severe gastroenteritis in infants and children under the age of five in the world. It is estimated that rotavirus is responsible for 500,000 deaths a year worldwide among children under the age of five.[2-4] Almost every child has been infected with rotavirus by the age of five.[5] When children under the age of three are infected, they develop severe gastroenteritis and dehydration. Symptoms include mild to severe vomiting, mild to severe diarrhea, fever, headache, anorexia, malaise, abdominal cramps, gas, electrolyte imbalance, shock and death.[3,18] Upon infection rotavirus has an incubation period of 1-3 days with abrupt onset of symptoms which generally last seven days.[3,18] Worldwide, by the age of five, one in five children require medical

assistance for rotavirus induced gastroenteritis, one in 65 is hospitalized, and one in 293 dies.[5] Children from the poorest regions of the world account for 82% of all rotavirus deaths.[6]

Rotavirus is spread directly through person-to-person contact (fecal-oral route), although there is some speculation that airborne transmission is also possible.[3,19] It has been termed "winter gastroenteritis" since outbreaks normally peak during the colder months and are lowest during the warmer months of the year. In the U.S., most outbreaks occur in day care centers, schools, and pediatric hospitals. In poorer or less developed countries, outbreaks can occur within communities, villages and hospitals.[20]

The first rotavirus infection in children is usually the most severe, and deaths from rotavirus gastroenteritis are mostly attributed to initial infection. After an initial infection with rotavirus, approximately 88% of individuals are protected against severe gastroenteritis with a second infection.[3] Subsequent infections, usually asymptomatic, result in progressively higher rates of protection against severe disease.[3,21]

2.2.1 Rotavirus Vaccines

Recently, two new rotavirus vaccines have become available for use in infants in the U.S. called RotaTeq® and Rotarix®. RotaTeq® is the oldest and most widely used of the two vaccines in the United States. This vaccine has been shown to drastically reduce the number of cases of severe rotavirus gastroenteritis by about 98% while not causing any significant side effects in the patients.[3] This vaccine is widely available for use in the U.S. but at about $167 is too costly for widespread use in poorer countries or with uninsured patients in this country. Another issue is that RotaTeq® must be administered as three separate doses of vaccine starting at around two months of age. Ideally the doses should be administered at two, four, and six months of age and they must be spaced by 4-10 week intervals.[3] All three doses should be administered by 32 weeks of age.[3] There are insufficient data on the efficacy of vaccination with RotaTeq® after 32 weeks, so it is not recommended.[3] Rotarix® is not much different than RotaTeq® in price or dosing, except it only requires two necessary doses.

However, these doses must be given on a rigid time schedule similar to the RotaTeq® schedule. So the cost and rigid administration regime for the vaccines are prohibitive in most underdeveloped countries for widespread use at this time. This is especially concerning since recent data suggest that clean water supplies and good hygiene have minimal, if any, effect on virus transmission.[3] Improving the water quality in underdeveloped nations probably would not have any marked effect on rotavirus outbreaks. In one study, it was shown that when rotavirus virions were placed on fingertips, after 60 minutes, 43% of the virions had survived on the fingertips and were potentially infectious if transmitted.[18] This study revealed that extremely thorough hand washing is necessary to avoid rotavirus outbreaks, but is that likely to occur in impoverished regions or even in the United States? Also, not all disinfectants will inactivate rotavirus virions. Phenolic disinfectants are useless against the virus.[18] Even though the costs of the vaccines are high and their administration schedules are limited, RotaTeq® and Rotarix® appear to be the best options at this time to help alleviate unnecessary gastrointestinal illness induced by rotavirus in impoverished infants and toddlers.

Realizing that global mass vaccination of infants with the RotaTeq® and Rotarix® vaccines is unlikely, I am looking at alternative treatment options to prevent or alleviate symptoms of rotavirus infections.

2.3 Likelihood of Co-infections

Since these two viruses, rotavirus and reovirus, are so ubiquitous and almost all individuals have antibodies against them, by the age of five, it is likely that co-infection with these two viruses occur in nature. This theory can be supported by the route of transmission of both of the viruses. Both viruses are known to be transmitted person-to-person by the fecal-oral route so it is likely that they could be transmitted concurrently. The fact that reovirus has been found in healthy and ill individuals also makes it likely that an individual suffering from rotavirus infection could also transmit, unknowingly, reovirus even though no additional symptoms of another illness are present. It has long been documented that co-infections with various enteric

viruses occurs naturally in humans and animals.[30,40-42] Co-infections that occur naturally are likely the result of superinfection. In virology, the phenomenon of superinfection occurs when an individual already infected with one type of virus is then infected with a second type of virus.

It has been reported that during co-infection experiments using BS-C-1 cells (African green monkey kidney cells), simian rotavirus (SA11) inhibits reovirus MRV-3DE production of infectious particles.[22] As an investigation into this claim, I performed a co-infection experiment as the beginning of this project using SA11 and MRV-3DE in MA104 cells (African green monkey kidney cells) and obtained the opposite results. Reovirus MRV-3DE was unaffected by SA11, while SA11 was inhibited by the presence of MRV-3DE. These results led me to develop an experimental approach to investigate whether inhibition of rotavirus occurred with all three serotypes of reovirus and whether rhesus rotavirus (RRV) was similarly inhibited by all three reovirus serotypes.

2.4 Viruses, Cell Lines, and Animals Used in this Study

2.4.1 Viruses used in this study

Two different types of viruses were used in this study: Mammalian orthoreoviruses and rotaviruses. Two rotaviruses were used to evaluate outcomes of co-infections in this study. The two rotaviruses are Simian Rotavirus (SA11, ATCC# VR-1565) (Figure 2.1) and Rhesus Rotavirus (RRV, ATCC# VR-954) (Figure 2.2). A total of 24 mammalian orthoreoviruses were used to evaluate outcomes of co-infections in this study. The three human reovirus serotypes previously discussed: MRV-1LA, MRV-2JO, and MRV-3DE (originally obtained from the laboratory of Dr. W.K. Joklik, 1995) (Figure 2.3) were used, as well as, two sets of mammalian orthoreovirus monoreassortants (10 viruses per set) (Figures 2.4 and 2.5).[23,24] All of the rotaviruses and reoviruses are nonenveloped double stranded RNA (dsRNA) viruses that replicate in the cell's cytoplasm.

Reovirus monoreassortants are viruses that each contain one genome segment of one parental serotype in the genetic background of another parental serotype.[23] These

monoreassortants have, historically been useful in identifying the proteins responsible for several stages of the reovirus replication cycle.[23] The sets of monoreassortants used were MRV-1LA with MRV-3DE genes (ST1wST3) and MRV-3DE with MRV-2JO genes (ST3wST2). Each set includes ten viruses that each contains nine gene segments from one reovirus serotype and one gene segment from another reovirus serotype. Each virus in the ST1wST3 set contains nine genes from reovirus MRV-1LA and one gene from reovirus MRV-3DE. Each virus in the ST3wST2 set of monoreassortants contains nine genes from reovirus MRV-3DE and one gene from reovirus MRV-2JO. For example, ST1wST3 L1 contains the L1 gene from reovirus MRV-3DE and the remaining nine genes (L2, L3, M1, M2, M3, S1, S2, S3, and S4) from reovirus MRV-1LA. These monoreassortants were created by injecting L929 cells with mixtures of viral cores (not just the viral RNA) of the two serotypes of interest.[23] Usually about 30 cores of MRV-2JO and only 2 cores of MRV-3DE were injected into the cells.[23] This would result in about 14% of the progeny viruses being monoreassortants containing one gene segment from MRV-2JO and 9 gene segments from MRV-3DE.[23] The same process was used to construct the set of ST1wST3 monoreassortants.[23] The resulting progeny viruses from the infections were plaque purified and the parental origins of all genome segments in the reassortants was determined through electrophoresis.[23] The progeny genes were compared to the parental viral genes to determine the genetic makeup of each progeny virus.[23] In addition to visual comparison to determine parental gene origin, the inserted genes were sequenced to confirm parental origin.

The reovirus serotypes MRV-1LA, MRV-2JO, and MRV-3DE were used for this project because they are the only serotypes of reovirus known to infect humans. The two sets of reovirus monoreassortants used were chosen because they are merely human serotypes that have reassorted. Rhesus rotavirus was used as the main rotavirus for the study for several reasons. One, it is extremely difficult to grow human rotavirus in the lab. Rhesus rotavirus is so similar to the human rotavirus that it was actually the strain used in one of the first human

rotavirus vaccines. Next, rhesus rotavirus is relatively easy to grow in the lab. It, also, readily infects mice and consistently results in diarrhea upon infection. The SA11 simian rotavirus was chosen because it readily infects many animals in nature and I wanted to determine if any of the three human reovirus serotypes would have an inhibitory effect against another very common non-human rotavirus serotype.

2.4.2 Cell lines used in this study

MA104 cells, African green monkey kidney cells, (ATCC# CRL-2378) were used in this study because they are the standard cell line used in rotavirus research. They are easily grown and maintained in the lab, as well. The MA104 cells were used for all rotavirus infections and the co-infections. They were chosen for use with the co-infections because they are readily infected by both rotavirus and reovirus. L929 cells, mouse fibroblasts cells, (ATCC# CCL-1) were used with all reovirus-only infections because they are the standard cell line used in reovirus research. They were not used for the actual co-infections because my studies showed that they are not as readily infected by rotavirus as MA104 cells.

2.4.3 Animals used in this study

BALB/c mice were chosen to test the effectiveness of MRV-1LA, MRV-2JO, and MRV-3DE at preventing or lessening the diarrhea produced from RRV infections for several reasons. Firstly, they are the most cost effective mammal model for this study out of all the options (i.e. pigs, rabbits, and humans). Pigs would have been the optimal animal to use because they respond to rotavirus infection most like humans out of all the other possible animal choices but they are extremely expensive in time, effort, space, and financial respects to maintain. Also, their high level of intelligence makes it difficult to get pigs approved as a “first-line” research animal. Rabbits are too expensive and require more space than what was available for this project. Also, they don’t develop diarrhea as readily as mice and pigs do in response to rotavirus infection. Human children were not an option for this study.

BALB/c mice were chosen as the mammal model for this study for several non-financial reasons, as well. The mice were readily available and large numbers were easy to acquire through breeding in the lab. This type of mouse is easily infected by the rhesus rotavirus strain used in this study and quickly and consistently develops diarrhea in response to infection. They require relatively little space to house and maintain in the lab and they are easy to handle, weigh, and check for diarrhea on a daily basis.

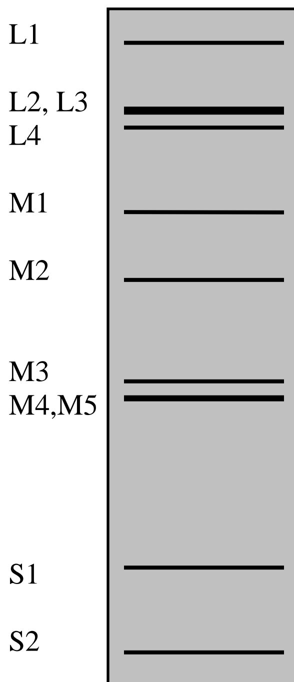

Figure 2.1 Simian Rotavirus (SA11) gene segments. (L = large gene segment, M = medium gene segment, S = small gene segment)

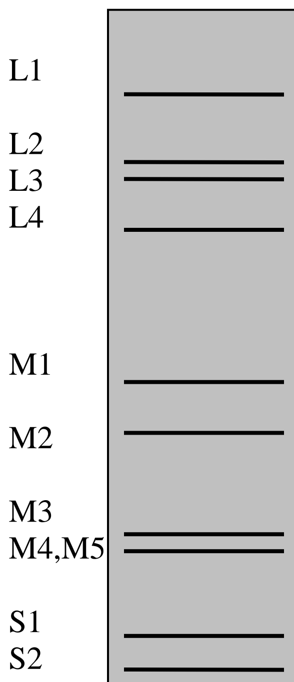

Figure 2.2 Rhesus Rotavirus (RRV) gene segments. (L = large gene segments, M = medium gene segments, S = small gene segments)

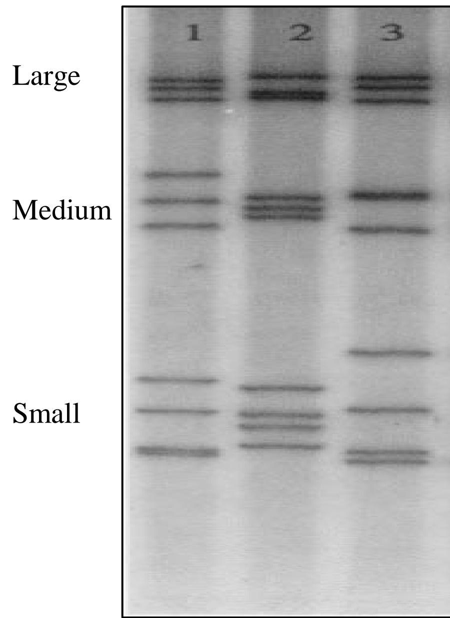

Figure 2.3 Mammalian Orthoreovirus serotypes. (1) MRV-1LA, (2) MRV-2JO, and (3) MRV-3DE[26] (Large = large genome segments, Medium = medium genome segments, Small = small genome segments)

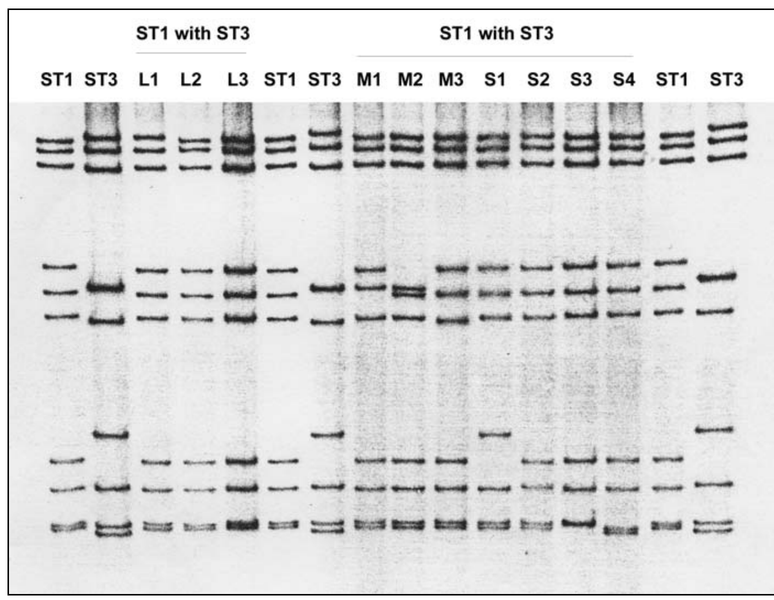

Figure 2.4 Analysis of the genome segments in the ST1wST3 monoreassortants [24]

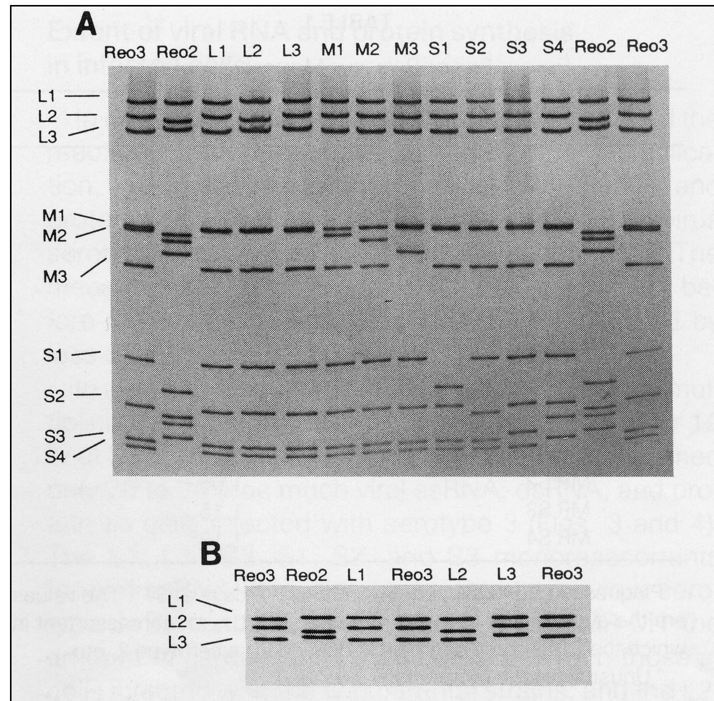

Figure 2.5 Analysis of the genome segments in the ST3wST2 monoreassortants [23]

2.4.4 Overview of Rotavirus and Reovirus Replication

Both viruses attach and enter the host cell's cytoplasm via receptor-mediated endocytosis. The virus particles are then partially uncoated and consist of inner-capsid subviral particles that contain 10 or 11 gene segments (reovirus has 10 and rotavirus has 11 gene segments) and transcriptional enzymes. The subviral particles then begin manufacturing viral mRNA using virally-coded RNA polymerase packaged in the virion. The viral mRNAs are then capped and methylated by viral enzymes and extruded through the vertices of the viral capsid. Next the mRNA is translated into amino acids that begin to form a new inner capsid. An unknown sequence of events ensures that exactly one copy of each gene segment is inserted into the newly formed capsid. Inside the newly formed capsid, the positive-sense mRNAs are then used as templates for the synthesis of negative-sense mRNA. New virus particles are formed when the immature capsid buds into the lumen of the endoplasmic reticulum.[28] Once enough virus particles are formed in a cell, they are released through cell lysis (Figure 2.6).

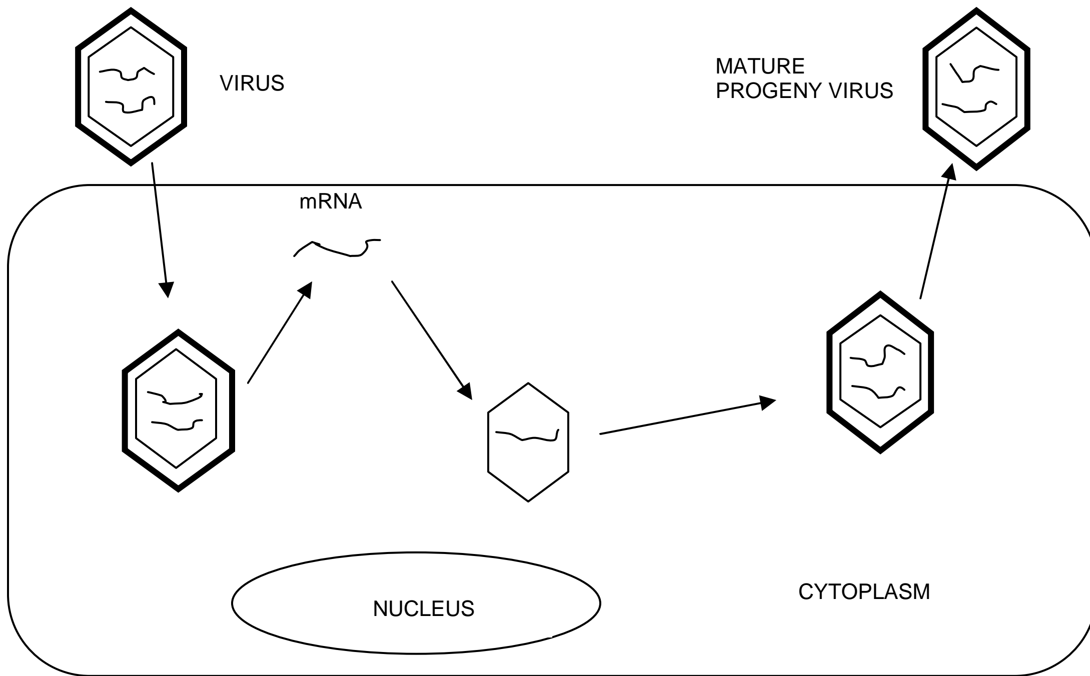

Figure 2.6 Overview of rotavirus and reovirus replication

CHAPTER 3

MATERIALS AND METHODS

3.1 Cell Maintenance

The cell lines used were L929 and MA104 , which were cultured in cell culture flasks that allowed the cells to adhere to the bottom of the flask. The L929 (mouse fibroblast cells, ATCC# CCL-1) cell line was maintained in Dulbecco's Modification of Eagle's Medium (MEM), obtained from cellgro®, supplemented with bovine calf serum (BCS) at a final concentration of 5%, and the antibiotics penicillin [100 units/mL] and streptomycin [100 μg/mL] (APS) at a final concentration of 1%. The MA-104 (African green monkey kidney cells, ATCC# CRL-2378) cell line was maintained in MEM supplemented with fetal bovine serum (FBS) at a final concentration of 5%, and APS at a final concentration of 1%. All cells were incubated at 37°C with 5% CO_2.

When cell lines reached confluency in the cell culture flasks, they were split to seed necessary plates for experimentation and to propagate the cells for maintenance. Both cell lines were split by the same method. First the media was removed and the cells were washed with a 1X saline sodium citrate (SSC) solution. Then the 1X SSC was removed and trypsin was added to remove the cells from the bottom of the cell culture flask. After the cells were released from the bottom of the flask, the appropriate MEM media was added to the flask. The cells were pipetted up and down to break up large aggregates of cells and to mix the cells and media. The dilution ratio of the cells was then dependent on the need for the cells.

3.2 Preparation of Viral Stocks

The simian rotavirus (SA11) stock and the rhesus rotavirus (RRV) stocks were grown in MA104 cells. All the reovirus stocks (MRV-1LA, MRV-2JO, MRV-3DE, and two sets of

monoreassortants) were grown in L929 cells. All virus stocks were grown in the same manner. Cell culture flasks (75 cm^2) approximately 80% confluent with the appropriate cells had all media removed. Then approximately 500μL to 1000μL of virus stock was added to the flask. The flask then had 1000μL to 1500μL of MEM without serum added to it, to make the final volume in the flask 2000μL. The MEM added to the flasks with rotavirus stocks contained 250 μg/mL of trypsin. The MEM added to the flasks with reovirus stocks did not contain any trypsin. The flask was allowed to incubate for one hour with rocking every 15 minutes at 37℃ and 5% CO_2. Then, enough MEM was added to the flask to bring the final volume up to 10mL. Once again, the MEM added to the rotavirus stocks contained 250 μg/mL of trypsin. The flasks were then incubated at 37℃ and 5% CO2 until approximate ly 80% of the cells were dead. This usually occurred after one to seven days of incubation. The flasks were then sonicated and the contents were stored in screw cap vials at -20℃ un til further use. Standard plaque assays were performed on the stocks to determine the amount of virus particles per milliliter of stock (the plaque forming units per milliliter (pfu/mL)).

3.3 Rhesus Rotavirus and Reovirus Co-infection Evaluations

3.3.1 Rhesus Rotavirus and Reovirus MRV-1LA, MRV-2JO, and MRV-3DE Co-infections

MA104 cells were plated at 80% confluency in six well plates. After an overnight incubation at 37℃ and 5% CO_2 to allow the wells to become 100% confluent with cells, the media was removed. Then the MA-104 cells were infected with RRV at a multiplicity of infection (MOI) of 10 in MEM, without serum, which contained 250μg/mL trypsin. An MOI of 10 meant that there were 10 virus particles per every one cell in the media. Next, the appropriate amount of MRV-1LA, MRV-2JO, or MRV-3DE diluted in MEM, without serum and which contained 250μg/mL of trypsin, at a MOI of 10 was also added to the appropriate wells. The wells then had enough MEM, which containing 250μg/mL of trypsin but no serum, added to bring the final volume in each well to 300μL (Figure 3.1). Co-infections were performed in triplicate (three wells). Single infection controls of RRV and the appropriate reovirus serotype were performed

in duplicate (two wells each) (Figure 3.2). All wells were brought to a final volume of 300µL with MEM, no serum, which contained 250µg/mL. After one hour of incubation at 37℃ and 5% CO_2 with rocking every 15 minutes, the inoculum and media were removed. Then three milliliters of MEM with 5% FBS and 125µg/mL of trypsin were added to each well. The plates were then incubated at 37℃ and 5% CO_2 for 72 hours. After 72 hours, the wells were all sonicated and the contents were collected individually and stored at -20℃ until use.

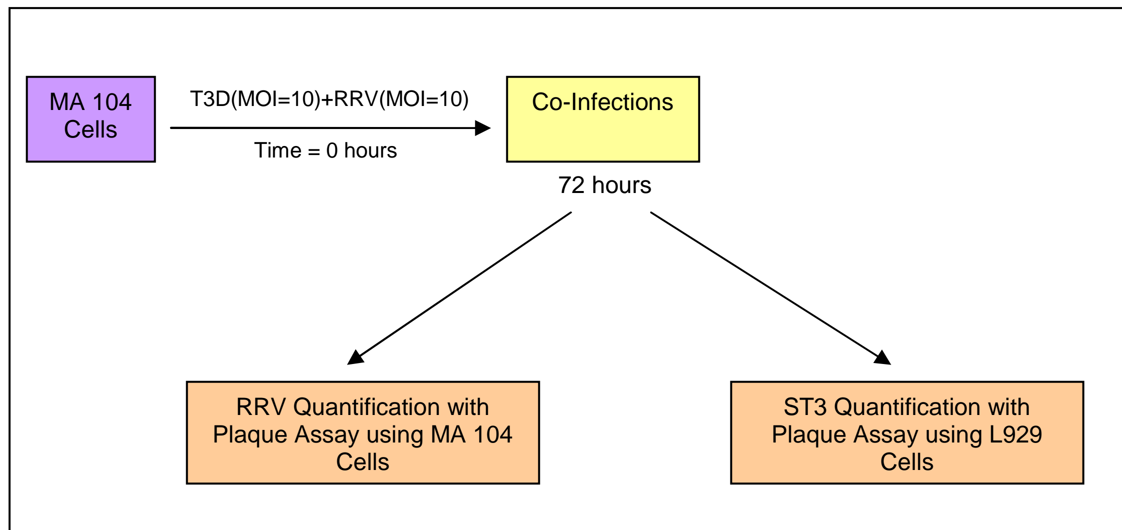

Figure 3.1 Summary of rhesus rotavirus and MRV-3DE (T3D) co-infections. (MRV-1LA and MRV-2JO co-infections performed in same manner)

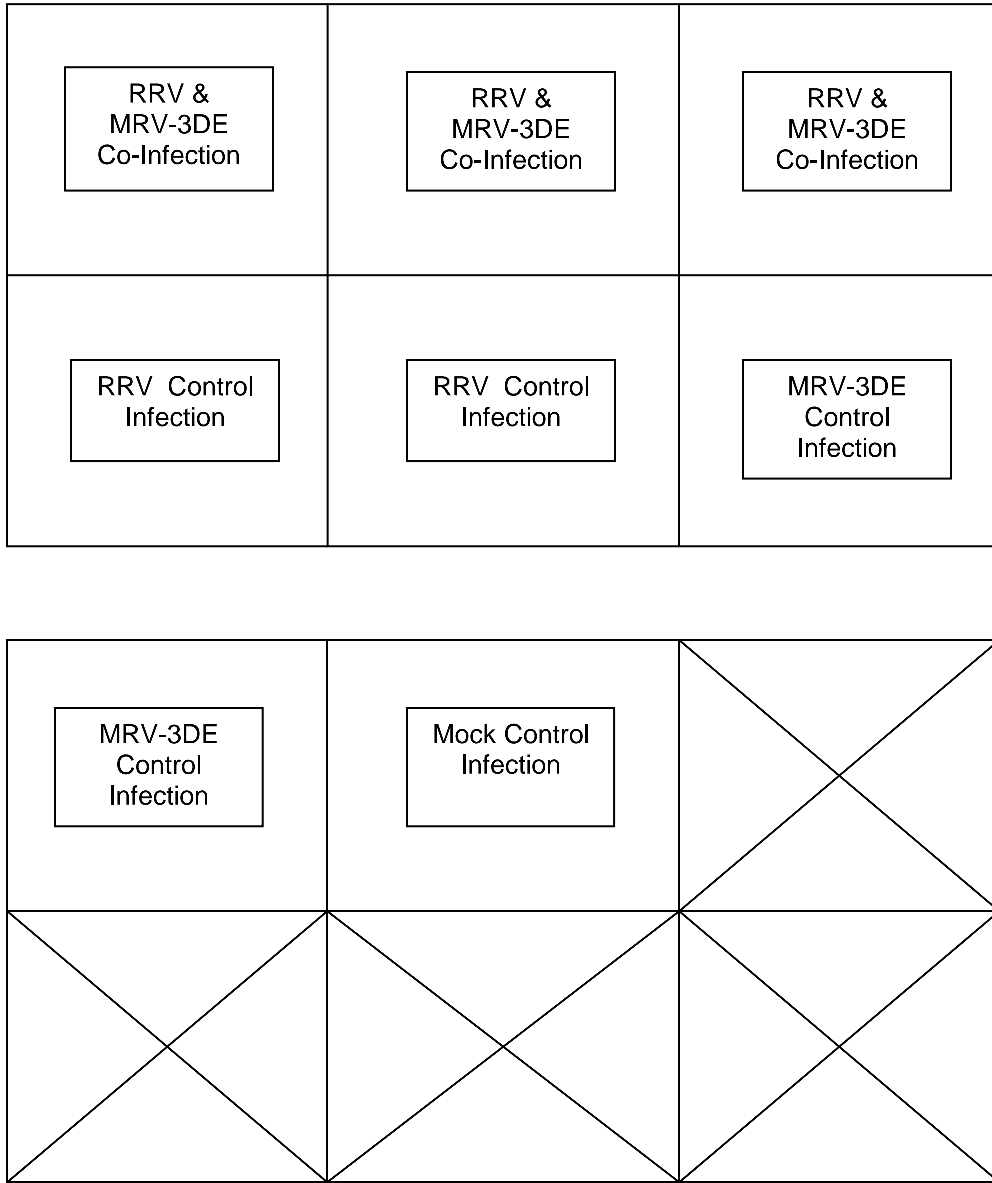

Figure 3.2 Diagrams of plates of RRV and MRV-3DE co-infections

3.3.2 Rhesus Rotavirus and Reovirus MRV-3DE Superinfections

The effect of viral superinfection with RRV and MRV-3DE was studied. MA-104 cells were plated at 80% confluency in six well plates. The plates were incubated overnight at 37℃ and 5% CO2 to become 100% confluent with cells. After incubation, the media was removed and the wells were infected with MRV-3DE for 1, 2, 4, or 6 hours at a MOI of 10. The plates

were incubated at 37°C and 5% CO2 with rocking every 15 minutes during the 1, 2, 4, or 6 hours of infection. Then after the appropriate 1, 2, 4, or 6 hours of incubation, the MRV-3DE inoculum was removed and enough RRV was added to the wells for a MOI of 10. The plates were then incubated at 37°C and 5% CO2 for one hour with rocking every 15 minutes. Then the RRV inoculum was removed and three milliliters of MEM with 5% FBS, 1% APS, and 250μg/mL of trypsin was added to each well. The plates were incubated at 37°C and 5% CO2 for 72 hours (Figure 3.3). The contents of the wells were then sonicated and stored at -20°C until further use.

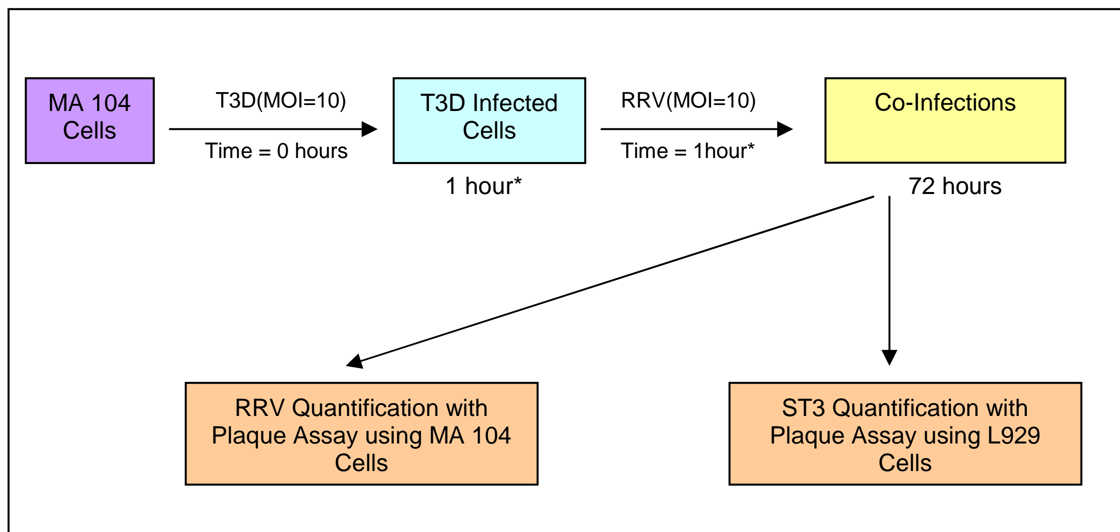

Figure 3.3 Summary of RRV superinfection infection scheme. (*the 2, 4, and 6 hour superinfections were performed in the same manner except an initial 2, 4, or 6 hours elapsed before the RRV was added to the cells)

The opposite superinfection scenario also was studied. Six well plates 100% confluent with MA104 cells were infected with RRV at a MOI of 10. The plates were incubated for 1, 2, 4, or 6 hours at 37°C and 5% CO2. After the appropriate incubation, the RRV inoculum was removed. Next MRV-3DE was used to infect the cells at a MOI of 10 for one hour at 37°C and 5% CO2. The MRV-3DE inoculum was then removed and three milliliters of MEM with 5% FBS, 1% APS, and 250μg/mL of trypsin was added to each well. The plates were incubated for 72

hours at 37℃ and 5% CO2 (Figure 3.4). After incub ation, the contents of the wells were sonicated and stored at -20℃ until further use.

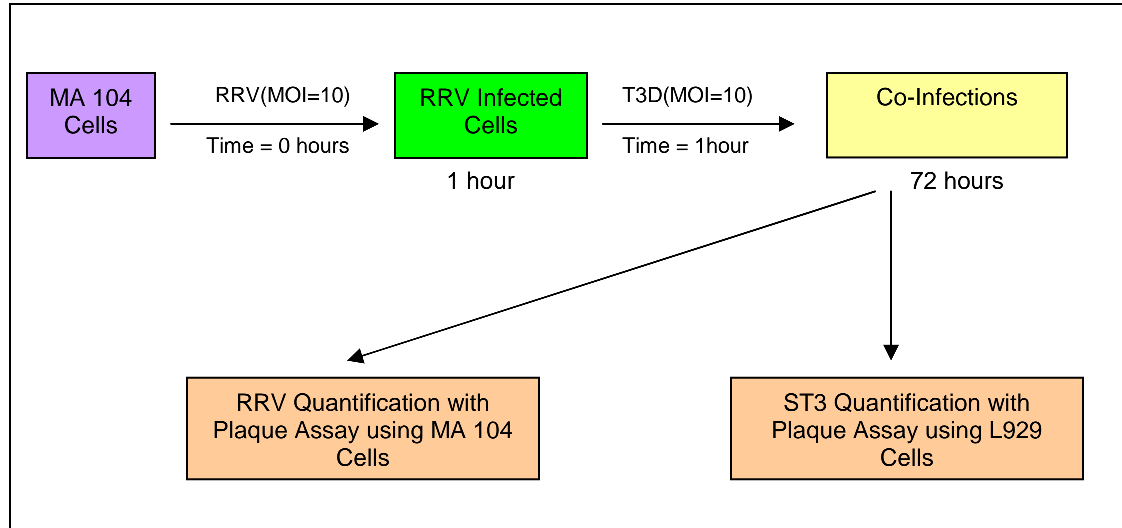

Figure 3.4 Summary of MRV-3DE (ST3) superinfection infection scheme. (*the 2, 4, and 6 hour superinfections were performed in the same manner except an initial 2, 4, or 6 hours elapsed before the ST3 was added to the cells)

Each type of superinfection was performed in triplicate and single control infections were performed in duplicate.

3.3.3 Rhesus Rotavirus and Reovirus Monoreassortant Co-infections

MA-104 cells were plated at 80% confluency in six well plates. After an overnight incubation at 37℃ and 5%CO_2 to allow the wells to become 100% confluent with cells, the media was removed. Then, the MA104 cells were infected with RRV at a multiplicity of infection (MOI) of 10 in MEM, without serum, which contained 250μg/mL trypsin. Next, the appropriate amount of each of the ten viruses from both sets of orthoreovirus monoreassortants (ST1wST3 and ST3wST2 sets) diluted in MEM (containing 250 μg/mL of trypsin but no serum) at a MOI of 10 was also added to the appropriate wells. The wells then had enough MEM, without serum, which contained 250μg/mL of trypsin added to bring the final volume in each well to 300μL. Co-infections were performed in three wells. Single infection controls of RRV and the appropriate reovirus monoreassortants were performed in two wells each. All wells were brought to a final

volume of 300µL with MEM (containing 250µg/mL of trypsin but no serum). After one hour of incubation at 37℃ and 5% CO_2 with rocking every 15 minutes, the inoculum and media were removed. Then three milliliters of MEM with 5% FBS and 125µg/mL of trypsin was added to each well. The plates were then incubated at 37℃ and 5% CO_2 for 72 hours (Figures 3.5 and 3.6). After 72 hours, the wells were all sonicated and the contents were collected individually and stored at -20℃ until use.

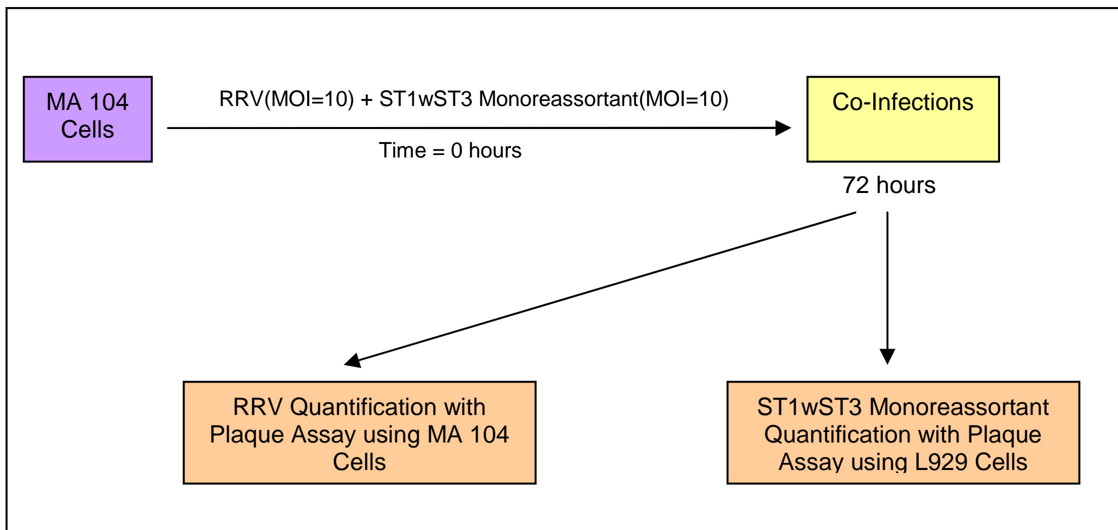

Figure 3.5 Summary of RRV and ST1wST3 monoreassortant co-infections

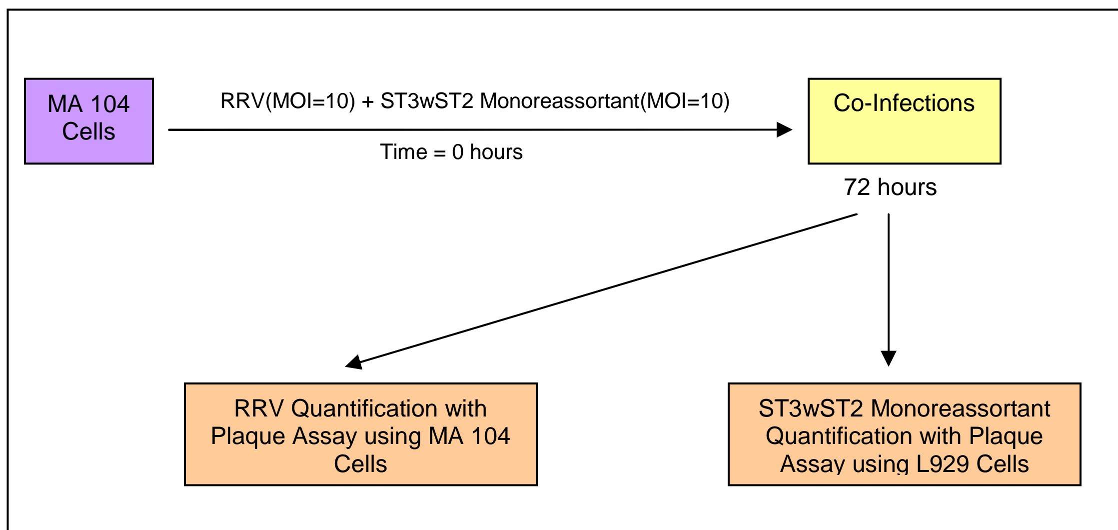

Figure 3.6 Summary of RRV and ST3wST2 monoreassortant co-infections

3.3.4 Reovirus Quantification

The amount of infectious reovirus produced during all co-infections was quantified by plaque assay. The plaque assays for reovirus MRV-1LA, MRV-2JO, MRV-3DE, and all twenty monoreassortant viruses were performed by the following method. The plaque assays were performed in 12-well plates that were 100% confluent with L929 cells, a cell line in which reovirus is lytic. All media was removed from the confluent wells and 125μL of the appropriate reovirus at serial 10-fold dilutions in MEM without serum was added to the appropriate wells. The plates were then incubated at 37℃ and 5% CO_2 for one hour with rocking every 15 minutes. The inoculum was removed from each well and two milliliters of a 50:50 agar and MEM solution was added to each well. The agar solution contained 50% of a 2% Noble agar and 50% of MEM with 10% BCS and 1% APS. Every 48 hours, another 2mL of agar-MEM solution was added to each well. Plates remained in incubation in between additions of agar solution. After four days for the MRV-1LA, MRV-3DE, and the ST1wST3 monoreassortant viruses another 2mL of agar-MEM solution that contained 3-4% of neutral red (a viability stain actively transported and sequestered into living cells) was added to each well. The MRV-2JO and the ST3wST2 monoreassortant viruses had the agar-MEM solution containing 3-4% of neutral red added to each well after an initial 6 days of incubation instead of 4 days for MRV-1LA, MRV-3DE and the ST1wST3 monoreassortants. After the addition of the agar solution containing neutral red, the plates were again incubated for 24-48 hours (long enough for the neutral red to properly stain the living cells) and then examined. Live cells were stained red allowing the plaques (small clear circles of dead cells) to be easily counted.

The plaque forming units (number of infectious virus particles) per milliliter of virus stock was then calculated as follows:

pfu/mL = [# of plaques in a well / (dilution factor of virus stock in well * 8)]

The dilution factor is multiplied by eight because the initial amount of virus dilution added to the well was 125μL which is 1/8 of a milliliter, so 125μL multiplied by eight equals 1mL. The pfu/mL

is then multiplied by three milliliters to give a final plaque forming unit (pfu) number. The final pfu is the amount of infectious virus plaque forming units produced by the reovirus during the actual co-infections and control infections.

3.3.5 Rhesus Rotavirus Quantification

The amount of rotavirus produced during single and co-infections were determined with plaque assays, as well. MA104 cells, a cell line in which rotavirus is lytic, were placed in 12-well plates at about 80% confluency and were incubated at 37℃ and 5% CO_2 until 100% confluent (24-48 hours). All media was removed from the confluent wells and 125μL of the appropriate virus at serial 10-fold dilutions in MEM without serum and containing 250μg/mL trypsin was added to each well. Antiserum containing antibodies against reovirus was added to each well containing virus produced from co-infections at a 50:1 ratio to prevent reovirus plaque formation. After one hour of incubation at 37℃ an d 5% CO_2 with rocking every 15 minutes, the inoculum was removed. Two milliliters of a 50:50 agar and MEM solution was added to each well. The agar-MEM solution contained 50% of 2% Noble agar and 50% of MEM containing 5% FBS and 1% APS. The agar-MEM solution contained 125μg/mL of trypsin. The plates were then incubated at 37℃ and 5% CO_2. Every 48 hours another two milliliters of the agar-MEM solution was added to each well. After four days, two milliliters of the agar-MEM solution containing 3-4% of neutral red was added to the wells. The plates were incubated and examined after 48 hours. Live cells were stained red, allowing the clear plaques to be counted.

The plaque forming units (number of infectious virus particles) per milliliter of virus stock was then calculated as follows:

pfu/mL = [# of plaques in a well / (dilution factor of virus stock in well * 8)]

The dilution factor is multiplied by eight because the initial amount of virus dilution added to the well was 125μL which is 1/8 of a milliliter, so 125μL multiplied by eight equals 1mL. The pfu/mL is then multiplied by three milliliters to give a final plaque forming unit (pfu) number. The final

pfu is the number of infectious virus plaque forming units produced by the reovirus during the actual co-infections and control infections.

3.3.6 Analysis of Rhesus Rotavirus and Reovirus Co-infection Data

The RRV and reovirus data collected from the above co-infections were analyzed using unpaired two-tailed two-sample t-tests. This determined if there was any significant difference between the means of the amounts of the two viruses produced during co-infections compared to the amounts of virus produced during single infections.

3.4 Simian Rotavirus and Reovirus MRV-1LA, MRV-2JO, and MRV-3DE Co-infection Evaluations

3.4.1 Simian Rotavirus and Reovirus MRV-1LA, MRV-2JO, and MRV-3DE Co-infections

MA-104 cells were plated at 80% confluency in six well plates. After an overnight incubation at 37℃ and 5%CO_2 to allow the wells to become 100% confluent with cells, the media was removed. Then the MA104 cells were infected with SA11 at a multiplicity of infection (MOI) of 10 in MEM, without serum, which contained 250µg/mL trypsin. An MOI of 10 meant that there were 10 virus particles per every one cell in the media. Next, the appropriate amount of MRV-1LA, MRV-2JO, or MRV-3DE diluted in MEM, without serum and which contained 250µg/mL of trypsin, at a MOI of 10 was also added to the appropriate wells. The wells then had enough MEM, without serum, which contained 250µg/mL of trypsin added to bring the final volume in each well to 300µL. Co-infections were performed in three wells. Single infection controls of SA11 and the appropriate reovirus serotype were performed in two wells each. All wells were brought to a final volume of 300µL with MEM, no serum, which contained 250µg/mL. After one hour of incubation at 37℃ and 5% CO_2 with rocking every 15 minutes, the inoculum was removed. Then three milliliters of MEM with 5% FBS and 125µg/mL of trypsin was added to each well. The plates were then incubated at 37℃ and 5% CO_2 for 72 hours (Figure 3.7). After 72 hours, the wells were all sonicated and the contents were collected individually and stored at -20℃ until use.

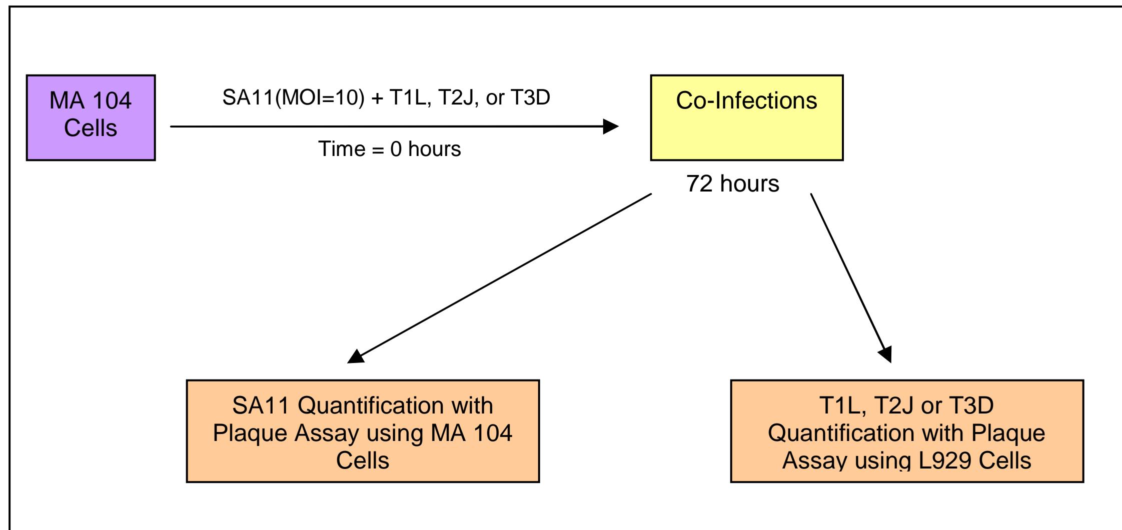

Figure 3.7 Summary of SA11 and MRV-1LA(T1L), MRV-2JO(T2J), or MRV-3DE(T3D) co-infections

3.4.2 Reovirus Quantification

The amount of infectious reovirus produced during all co-infections was quantified by plaque assay. The plaque assays for reovirus MRV-1LA, MRV-2JO, and MRV-3DE viruses were performed in the same following method. The plaque assays were performed in 12-well plates that were 100% confluent with L929 cells, a cell line in which reovirus is lytic. All media was removed from the confluent wells and 125µL of the appropriate reovirus serotype at serial 10-fold dilutions in MEM without serum was added to the appropriate wells. The plates were then incubated at 37℃ and 5% CO_2 for one hour with rocking every 15 minutes. The inoculum was removed from each well and two milliliters of a 50:50 agar and MEM solution was added to each well. The agar solution contained 50% of a 2% Noble agar and 50% of MEM with 10% BCS and 1% APS. Every 48 hours, another 2mL of agar-MEM solution was added to each well. Plates remained in incubation in between additions of agar solution. After four days for the MRV-1LA and MRV-3DE viruses another 2mL of agar-MEM solution that contained 3-4% of neutral red (a viability stain actively transported and sequestered into living cells) was added to each well. The MRV-2JO virus had the agar-MEM solution containing 3-4% of neutral red

added to each well after an initial 6 days of incubation instead of 4 days for MRV-1LA and MRV-3DE. After the addition of the agar solution containing neutral red, the plates were again incubated for 24-48 hours (long enough for the neutral red to properly stain the living cells) and then examined. Live cells were stained red allowing the plaques (small clear circles) to be easily counted.

The plaque forming units (number of infectious virus particles) per milliliter of virus stock was then calculated as previously explained.

3.4.3 Simian Rotavirus Quantification

The amount of rotavirus produced during single and co-infections were determined with plaque assays, as well. MA-104 cells, a cell line in which rotavirus is lytic, were placed in 12-well plates at about 80% confluency and were incubated at 37°C and 5% CO_2 until 100% confluent (24-48 hours). All media was removed from the confluent wells and 125µL of the appropriate virus at serial 10-fold dilutions in MEM without serum and containing 250µg/mL trypsin was added to each well. Antiserum containing antibodies against reovirus was added to each well containing virus produced from co-infections at a 50:1 ratio to prevent reovirus plaque formation. After one hour of incubation at 37°C and 5% CO_2 with rocking every 15 minutes, the inoculum was removed. Two milliliters of a 50:50 agar and MEM solution was added to each well. The agar-MEM solution contained 50% of 2% Noble agar and 50% of MEM containing 5% FBS and 1% APS. The agar-MEM solution contained 125µg/mL of trypsin. The plates were then incubated at 37°C and 5% CO_2. Every 48 hours another two milliliters of the agar-MEM solution was added to each well. After four days, two milliliters of the agar-MEM solution containing 3-4% of neutral red was added to the wells. The plates were incubated and examined after 48 hours. Live cells were stained red, allowing the clear plaques to be counted.

The plaque forming units (number of infectious virus particles) per milliliter of virus stock was then calculated as previously explained.

3.4.4 Analysis of Simian Rotavirus and Reovirus MRV-1LA, MRV-2JO, and MRV-3DE Co-infection Data

The SA11 and reovirus data collected from the above co-infections were analyzed using unpaired two-tailed two-sample t-tests. This determined if there was any significant difference between the mean amounts of the two viruses produced during co-infections compared to the amounts of virus produced during single infections.

3.5 *In Vivo* Co-infection Evaluations

3.5.1 In Vivo Animal Maintenance

The BALB/c mice were housed in micro-isolator cages and provided food and water ad libitum. The male and female breeding mice were acquired from two different sources to help prevent inbreeding. Once a female had been mated she was placed in her own isolator cage for the duration of her pregnancy. Each litter of mice (usually 6-8 mice) that were born was considered a group. All groups were allowed to nurse *ad libitum*. Each group was allowed to remain with their mother in the same cage until they were approximately 28 days old to ensure they were weaned and consuming only the provided commercial food and water. After approximately 28 days of age, the mother was removed from the cage and euthanized. All cages were changed/cleaned every seven days or sooner as needed. All mice in groups were labeled using non-toxic permanent marker on their backs.

3.5.2 In Vivo Infections

Each treatment was given to three separate groups. The control treatments consisted of MEM without serum, MRV-1LA, MRV-2JO, MRV-3DE, and RRV. The next set of treatments were co-infection treatments consisting of (1) MRV-1LA and RRV and (2) MRV-3DE and RRV. Each treatment was started on day six after birth for each group. Groups were inoculated with 25µL of the appropriate virus(es) (titers were 10^6 to 10^{14} pfu/mL). The treatments were gavage fed to the mice. The mice were fed the appropriate treatments for three consecutive days starting on day one of treatment (day six of life). For the groups that were used for co-

infections, RRV was gavage fed to the mice first and then immediately followed by the appropriate reovirus being gavage fed to the mice. One co-infection group was not given the viruses consecutively. This group was used to determine if the addition of MRV-1LA after rotaviral induced diarrhea began would lessen or shorten the duration of diarrhea. For this group, RRV was given to the three groups for three days beginning on day six of life as usual. Then after the mice developed diarrhea (on day two or three of treatment) they all began receiving MRV-1LA for days three, four, and five of treatment (Table 3.1).

The subjects were checked for diarrhea by gentle abdominal palpitations and were weighed daily for seven days starting on day one of testing. Stool was expressed, if possible, by gentle abdominal palpitations and then characterized as follows: zero, no stool expressed; one, formed brown stool expressed; two, brown soft stool expressed; three, yellow soft stool expressed; four, yellow liquid stool expressed.[43] For this evaluation, any stool rated three or four was considered diarrhea. Stool samples that were rated zero, one, or two were considered normal stool and not diarrhea. After the initial seven days of diarrhea checks and weight checks, all subjects were weighed and checked for diarrhea every seven days for 56 days. This was done to ensure that no lasting effects (i.e. growth/weight retardation, etc) had occurred to the mice from the treatments in comparison to control groups that will be fed MEM (a non-toxic sugar medium) only.

Table 3.1 Description of treatments given to mouse groups

Number of mouse groups	Treatment given to groups
3	MEM (mock control groups)
3	RRV
3	MRV-1LA
3	MRV-1LA and RRV co-infection (both viruses given at same time)
3	MRV-2JO
3	MRV-2JO and RRV co-infection (both viruses given at same time)
3	MRV-3DE
3	MRV-3DE and RRV co-infection (both viruses given at same time)
3	RRV given for 3days then MRV-1LA or MRV-2JO (which ever virus does the best lessening diarrhea in basic *in vivo* co-infections above) given until diarrhea stops to determine if MRV-1LA or MRV-2JO can stop/shorten diarrhea once infection has already occurred

3.5.3 Analysis of In Vivo Data

The weight data from the *in vivo* infections were analyzed using unpaired two-tailed two-sample *t*-tests.[29-35] This determined if there was any significant difference between the means of the weights from mice inoculated with MEM, single viruses and co-infections with two viruses. The diarrhea data (percent of individuals in groups that developed diarrhea) were analyzed with unpaired two-sample t-tests to determine if co-infections had significantly lower rates of diarrhea than single RRV infections.[29-36]

3.6 *In vitro* dsRNA Analysis

3.6.1 Infections for ^{32}P dsRNA Analysis and Electrophoresis

The amount of dsRNA produced by both RRV and ST1wST3 reovirus monoreassortants during single and co-infection *in vitro* situations was examined using SDS-Page gels. MA-104 cells were plated at 80% confluency in 24-well plates. After an overnight

incubation at 37℃ and 5%CO_2 to allow the wells to become 100% confluent with cells, the media was removed. Then the MA-104 cells were infected with RRV at a multiplicity of infection (MOI) of 10 in MEM, without serum, which contained 250μg/mL trypsin. Next, the appropriate amount of each of nine of the ST1wST3 monoreassortants (L1, L2, L3, M1, M2, S1, S2, S3, and S4) diluted in MEM, without serum and which contained 250μg/mL of trypsin, at a MOI of 10 was also added to the appropriate wells. The wells then had enough MEM (containing 250μg/mL of trypsin but no serum) added to bring the final volume in each well to 200μL. Co-infections were performed in duplicate wells. Single infection controls of RRV and the appropriate reovirus monoreassortants were performed in two wells each. All wells were brought to a final volume of 300μL with MEM, no serum, which contained 250μg/mL. After one hour of incubation at 37℃ and 5% CO_2 with rocking every 15 minutes, the inoculum and media were removed. Then 400μL of MEM with 5% FBS, 1% APS and 250μg/mL of trypsin was added to each well. The plate was then incubated at 37℃ and 5% CO_2 for about 12 hours.

After the initial 12 hours, each well had 100μL of ^{32}P solution added. The plate was then incubated for 60 additional hours at 37℃ and 5% CO_2. After the 60 hours of incubation, all media was removed from each well and 100μL of 1X Laemmli Sample Dye was added to each well. The plate was then frozen at -80℃ and thawe d at 100℃ four separate times.

3.6.2 dsRNA SDS-Page Electrophoresis

After the four rounds of freeze-thaws, 100μL of each sample was added to wells in a SDS-Page gel and electrophoresis was performed (Figure 3.8). The gel was a vertical gel for the Hoeffler apparatus that consisted of a 7.5% separating gel and a 4% stacking gel. The electrophoresis was performed using a 1X Laemmli running buffer. The gels were run for 2300 volt hours. After running, the gels were dried in a gel dryer for approximately 2 hours or until completely dried. They were then exposed to x-ray film for several weeks (approximately 2-12 weeks) before the films were developed.

Well #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Virus Added to Well	x	x	RRV	x	L1	L2	L3	M1	M2	S1	S2	S3	S4	x	x

Well #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Virus Added to Well	x	x	RRV	x	RRV / L1	RRV /L2	RRV /L3	RRV /M1	RRV /M2	RRV /S1	RRV /S2	RRV /S3	RRV /S4	x	x

Figure 3.8 ^{32}P SDS-PAGE gel layout. x=no sample added to well

3.6.3 Analysis of dsRNA Electrophoresis Data

The gels were scanned and analyzed using ImageJ computer software available from the NIH.[27] The data obtained was analyzed using unpaired two-tailed two-sample t-tests. This determined if there was any significant difference between the production of the dsRNA of the two viruses during co-infections in comparison to the viruses produced during single infections.

3.7 *In vitro* Protein Production Analysis

3.7.1 Infections for Protein Production Analysis

The amount of reovirus proteins produced during single and co-infection situations were examined using SDS-PAGE gels and electrophoresis. MA-104 cells were plated at 80% confluency in six-well plates. After an overnight incubation at 37°C and 5%CO_2 to allow the wells to become 100% confluent with cells, the media was removed. Then the MA104 cells were infected with RRV at a multiplicity of infection (MOI) of 10 in MEM, without serum, which contained 250µg/mL trypsin. Next, the appropriate amount of each of five of the ST1wST3 monoreassortants (L1, L3, S1, S2, and S3) diluted in MEM (containing 250µg/mL of trypsin but no serum), at a MOI of 10 was also added to the appropriate wells. The wells then had enough MEM, without serum, which contained 250µg/mL of trypsin added to bring the final volume in each well to 250µL. Co-infections were performed in duplicate wells. Single infection controls of RRV and the appropriate reovirus monoreassortants were performed in two wells each. After

one hour of incubation at 37℃ and 5% CO_2 with rocking every 15 minutes, the inoculum and media were removed. Then 1.5mL of MEM with 5% FBS, 1% APS and 250μg/mL of trypsin was added to each well. The plate was then incubated at 37℃ and 5% CO_2 for 72 hours. After incubation, all wells were scraped using a sterile cell scraper and contents of each well were placed in 1.5mL microcentrifuge tubes. The tubes were centrifuged at 2000rpm for 10 minutes to pellet the contents. The supernatant was removed and discarded. The pellet remaining in the tube was frozen at -20℃ until further use.

3.7.2 SDS-PAGE Gel Electrophoresis

The above pellets were thawed at room temperature for approximately 15 minutes or as needed. The pellets were then reconstituted with 100μL deionized H_20. Then 100μL of 2X Laemmli sample dye was added to each tube. The microcentrifuge tubes were then placed in a 100℃ water bath for approximately five minutes. T hen 40μL of each sample was added to the appropriate wells of a SDS-PAGE gel containing a 7.5% separating gel and a 4% stacking gel for Hoeffler apparatus (Figure 3.9). The gel was run at 35mA for 2.5 hours in 1X Laemmli running buffer. After all sample dye in the gel had run into the buffer at the bottom of the apparatus (approximately 2.5 hours of run time), the gels were removed from the apparatus.

Well #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Virus Added to Well	x	PM	RRV	L1	RRV /L1	L3	RRV /L3	S1	RRV /S1	S2	RRV /S2	S3	RRV /S3	PM	x

Figure 3.9 SDS-PAGE gel well layout for reovirus protein samples. PM=protein size marker 10μL, x=no sample added to well

3.7.3 Western Blot Procedure

After electrophoresis, the stacking gel portion of the gel was removed and discarded. The remaining separating gel was blotted onto a nitrocellulose membrane using a semi-dry transfer apparatus. The transfer ran with Towbin transfer buffer at 134mA for one hour. The gel had two wells that contained a colored protein marker that transferred to the membrane to confirm the semi-dry transfer process occurred without error. After the transfer the membrane was allowed to dry overnight at room temperature.

The dried membrane was then subjected to western blot analysis to determine the amounts of reovirus proteins produced during single and co-infections using SuperSignal® West Pico Chemiluminescent Substrate Kit. The membrane was first placed in approximately 50mL of Blocking Reagent for 60 minutes with rocking at room temperature. This ensured that nonspecific sites were blocked on the membrane. Then the Blocking Reagent was removed and approximately 50mL of the primary antibody solution was placed on the membrane. The membrane and primary antibody solution were allowed to incubate overnight at 4°C. The membrane was then washed six times for five minutes of agitation each time in Wash Buffer. The Wash Buffer was removed and the membrane was incubated with secondary antibody (Peroxidase Conjugated Goat anti-Rabbit IgG) for one hour with agitation at room temperature. The blot then was washed with Wash Buffer six times for five minutes each time with agitation at room temperature. Next the blot was incubated with Working Solution (equal parts Stable Peroxidase Solution and Luminol/Enhancer Solution) for five minutes at room temperature with gentle agitation. The blot was then removed from the working solution and placed in PVC wrap and exposed to x-ray film. The film was exposed to the blot for approximately five minutes and then developed.

3.7.4 Analysis of Reovirus Protein Production Data

Gels were scanned and analyzed using ImageJ computer software available from the NIH.[27]

CHAPTER 4

RESULTS

To determine if reovirus had the ability to significantly inhibit rotavirus during co-infections, several sets of *in vitro* and *in vivo* infections were performed. First, separate co-infections with RRV and MRV-1LA, MRV-2JO, MRV-3DE were performed to determine if each of the reovirus serotypes had the ability to inhibit infectious particle production by rotavirus in *in vitro* infections. Second, superinfections using RRV and MRV-3DE were explored to determine if the presence of RRV or MRV-3DE in cells for a time period of two to six hours before superinfection of MRV-3DE or RRV had an effect on the level of inhibition of infectious particle production of the viruses. Third, two sets of reovirus monoreassortants were used in co-infections with RRV to try to determine which reovirus gene or genes are responsible for the inhibition of rotavirus production. I also looked at SA11 co-infections with MRV-1LA, MRV-2JO, and MRV-3DE to determine if the inhibitions seen with RRV would occur with SA11. Next, we tried to determine the amounts of dsRNA produced by the viruses involved in the co-infections to see if there was an overall decrease in all genome segments in the inhibited viruses or a selected decrease in the production of single gene segments. If no differences were observed at the genome level, the possibility still existed that the inhibitory effect of one virus on another was due to an inhibition of synthesis of one or more viral proteins. To explore this possibility, the quantities of individual proteins produced by reovirus in certain co-infections was examined

I explored the outcome of *in vivo* infections to determine if our *in vitro* results would apply to mammals. We performed single and co-infections in mice using RRV and MRV-1LA, MRV-2JO, and MRV-3DE to determine if the presence of reovirus would lessen or inhibit the development of diarrhea in mice.

4.1 Rhesus Rotavirus and Reovirus MRV-1LA, MRV-2JO, and MRV-3DE Co-infections

Examining the impact of RRV on MRV during co-infections revealed that in the presence of RRV, the three serotypes of reovirus responded differently. MRV-1LA infectious particle production actually increased about 2-fold during co-infections with RRV in comparison to single MRV-1LA infections. MRV-2JO infectious particle production during co-infections was unaffected by the presence of RRV. MRV-3DE infectious particle production during co-infections was inhibited about 6-fold by the presence of RRV in comparison to single MRV-3DE infections (Figure 4.1 and Table A.1). Statistical analysis using t-tests revealed that MRV-1LA production was significantly enhanced by RRV during co-infections (p value = 0.003). MRV-2JO was not significantly affected by RRV during co-infections (p value = 0.956). MRV-3DE was significantly inhibited according to the t-test (p value = 0.000) by the presence of RRV during co-infections (Table A.2).

Using the same co-infections to explore the impact of MRV on RRV, showed that RRV was inhibited by MRV-1LA and MRV-2JO but completely unaffected by MRV-3DE (Figure 4.2 and Table A.3). Statistical analysis using t-tests revealed that RRV was significantly affected by the presence of MRV-1LA or MRV-2JO (p values = 0.000 and 0.000, respectively). RRV was not significantly (p value = 0.346) affected by the presence of MRV-3DE according to the t-test (Table A.4).

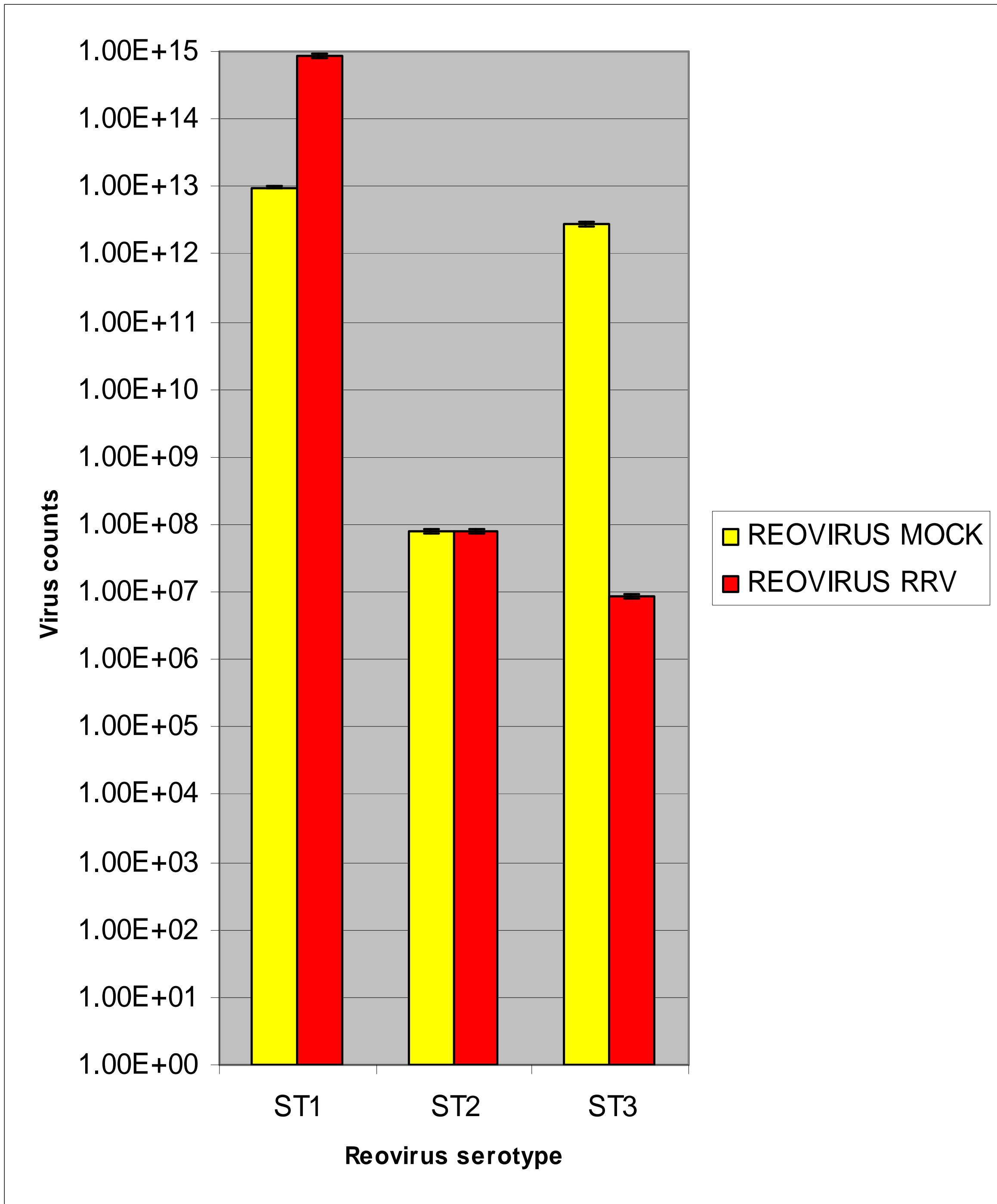

Figure 4.1 Reovirus recovery from rhesus rotavirus co-infections. (error bars represent 5% error)

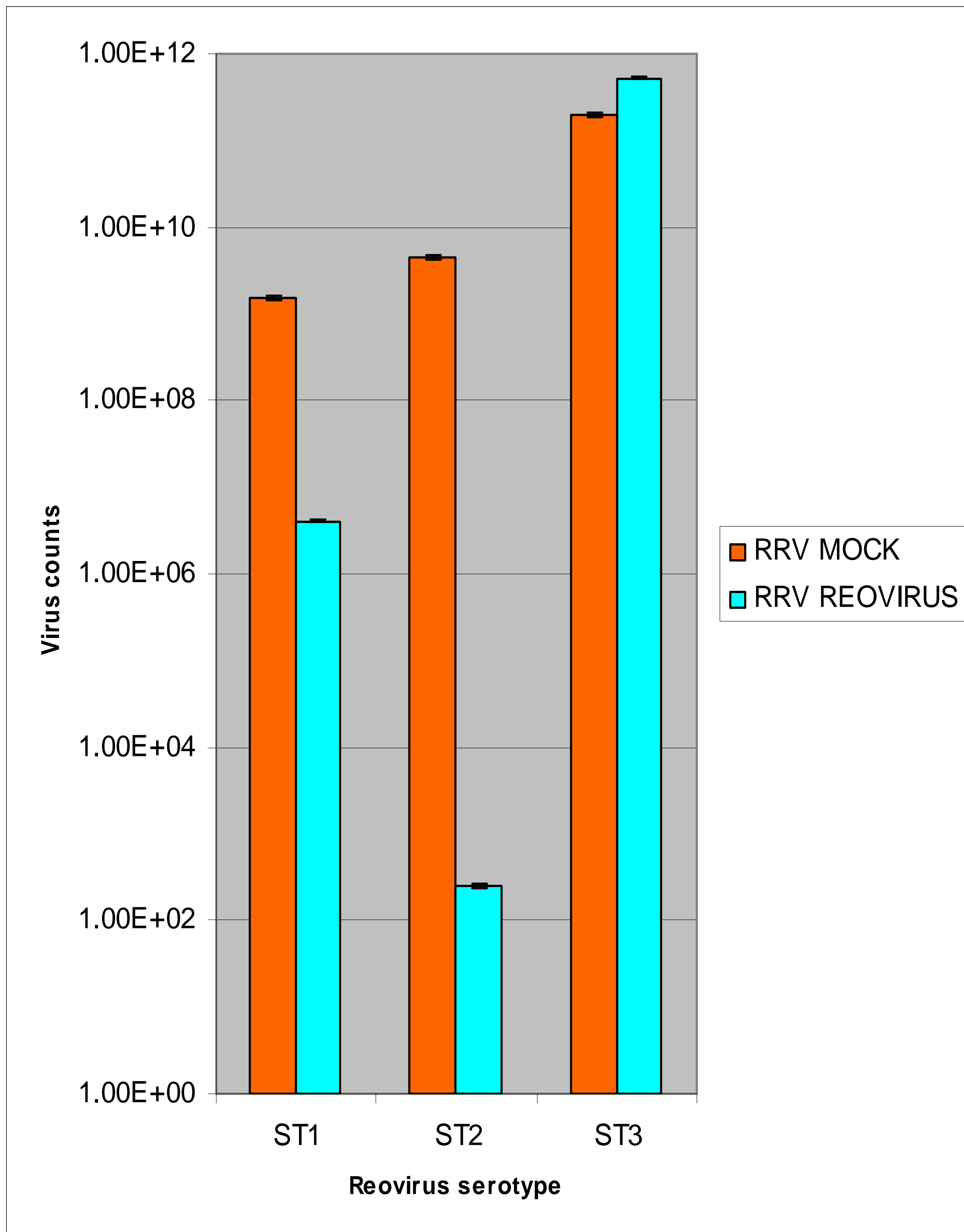

Figure 4.2 Rhesus rotavirus recovery from reovirus co-infections. (error bars represent 5% error)

4.2 Rhesus Rotavirus and Reovirus MRV-3DE Superinfections

4.2.1 Virus Recoveries from Rhesus Rotavirus Superinfections

Since superinfections readily occur in nature, the effects of RRV superinfections were studied. The impact on MRV replication was examined first. Cells were infected with MRV-3DE for one, two, four, or six hours before RRV was added to the cells. MRV-3DE replication appeared to be inhibited approximately two-fold regardless of when RRV was added to the cells. There was not a measurable difference in the amount of inhibition observed when RRV was added to cells previously infected with MRV-3DE for one, two, four, or six hours. Statistical analysis using t-tests support the observation that RRV did significantly inhibit MRV-3DE infectious particle production after its addition one, two, four, or six hours after MRV-3DE infection was initiated (*p* values = 0.000) (Figure 4.3 and Tables A.5 and A.6).

Using these same RRV superinfections, the impact on RRV by MRV was examined. Analysis of these superinfections revealed that RRV was inhibited seven-fold by the presence of MRV-3DE in cells for one, two, four, and six hours before RRV infects them (Figure 4.4 and Table A.7). Statistical analysis using t-tests showed that RRV was significantly inhibited by the presence of MRV-3DE in cells for one, two, four, and six hours before RRV superinfection (*p* values = 0.000, 0.001, 0.000, and 0.000, respectively) (Table A.8).

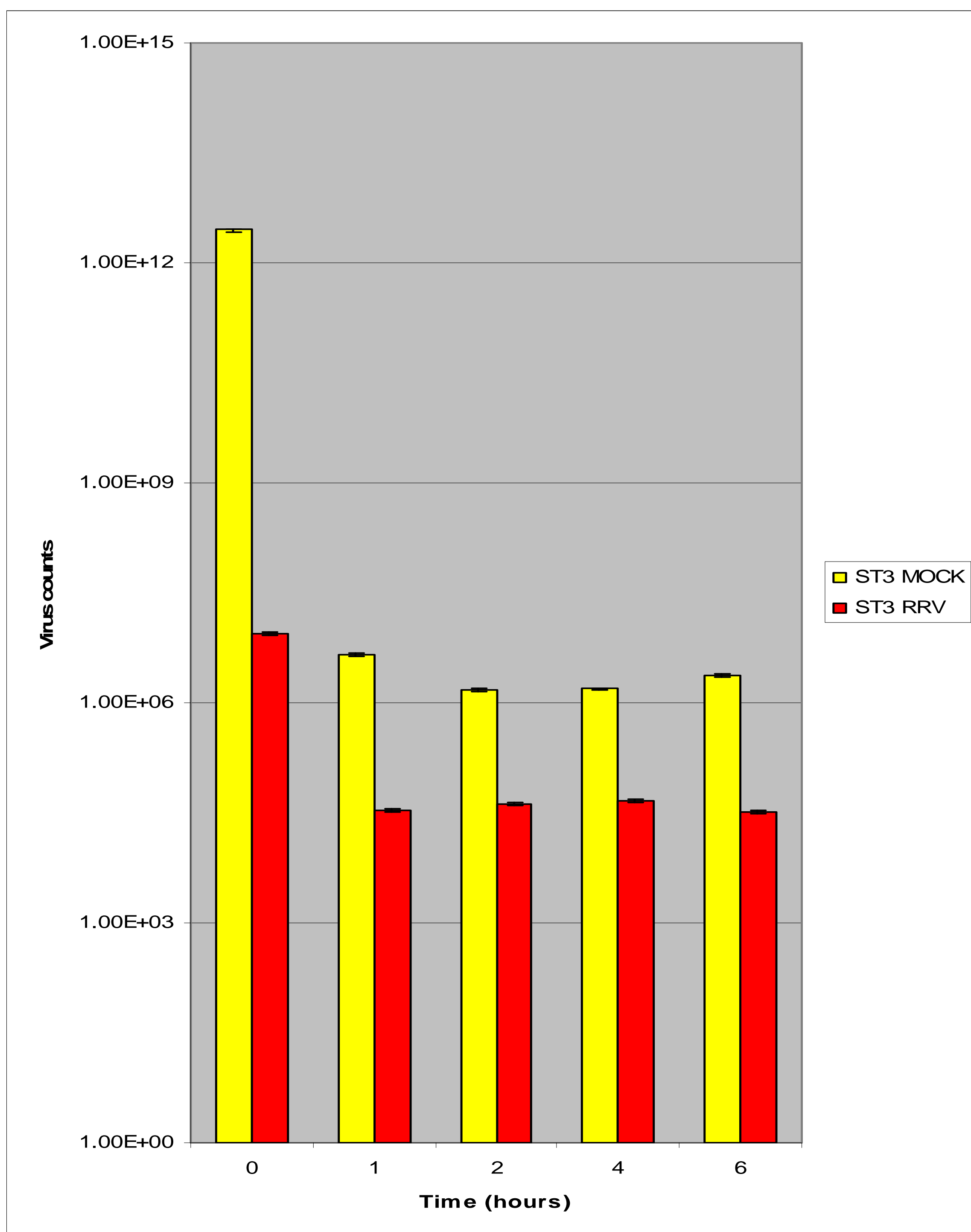

Figure 4.3 Reovirus ST3 (MRV-3DE) recovery from rhesus rotavirus superinfections. (error bars represent 5% error)

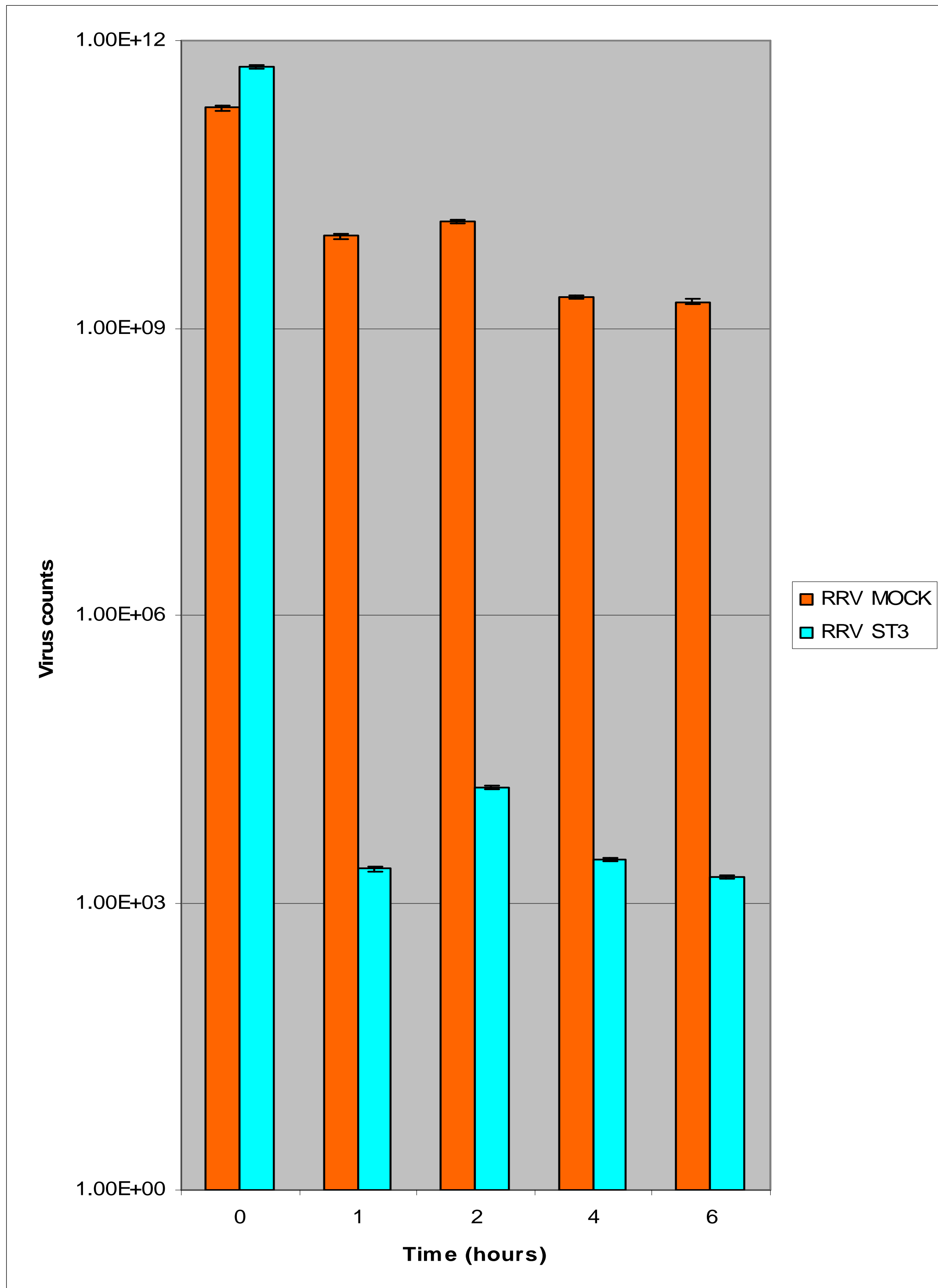

Figure 4.4 Rhesus rotavirus recovery from rhesus rotavirus superinfections. (error bars represent 5% error, ST3 = MRV-3DE)

4.2.2 Virus Recoveries from Reovirus MRV-3DE Superinfections

In addition to the effects of RRV superinfections, the effects of MRV-3DE superinfections were studied. MRV-3DE superinfections were performed after RRV had been in MA-104 cells for one, two, four and six hours. After the superinfections were incubated for 72 hours, it was evident that the presence of RRV still inhibited MRV-3DE in cells containing RRV for one or two hours prior resulted in a two-fold decrease of MRV-3DE production. The addition of MRV-3DE to cells already infected for four and six hours prior with RRV resulted in a complete inhibition of MRV-3DE infectious particle production (Figure 4.6 and Table A.9). T-tests revealed that the level of inhibition was significant (*p* value = 0.001 - 0.004) for the one, two, four, and six hour MRV-3DE superinfection, respectively (Table A.10).

These same MRV-3DE superinfections also revealed that RRV was inhibited approximately three- to four-fold by the addition of MRV-3DE to cells already infected with RRV for up to six hours (Figure 4.7 and Table A.11). T-tests showed that RRV was significantly inhibited by the addition of MRV-3DE to cells already infected with RRV for one, two, and six hours (*p* value = 0.002, 0.001, and 0.000, respectively) (Table A.12).

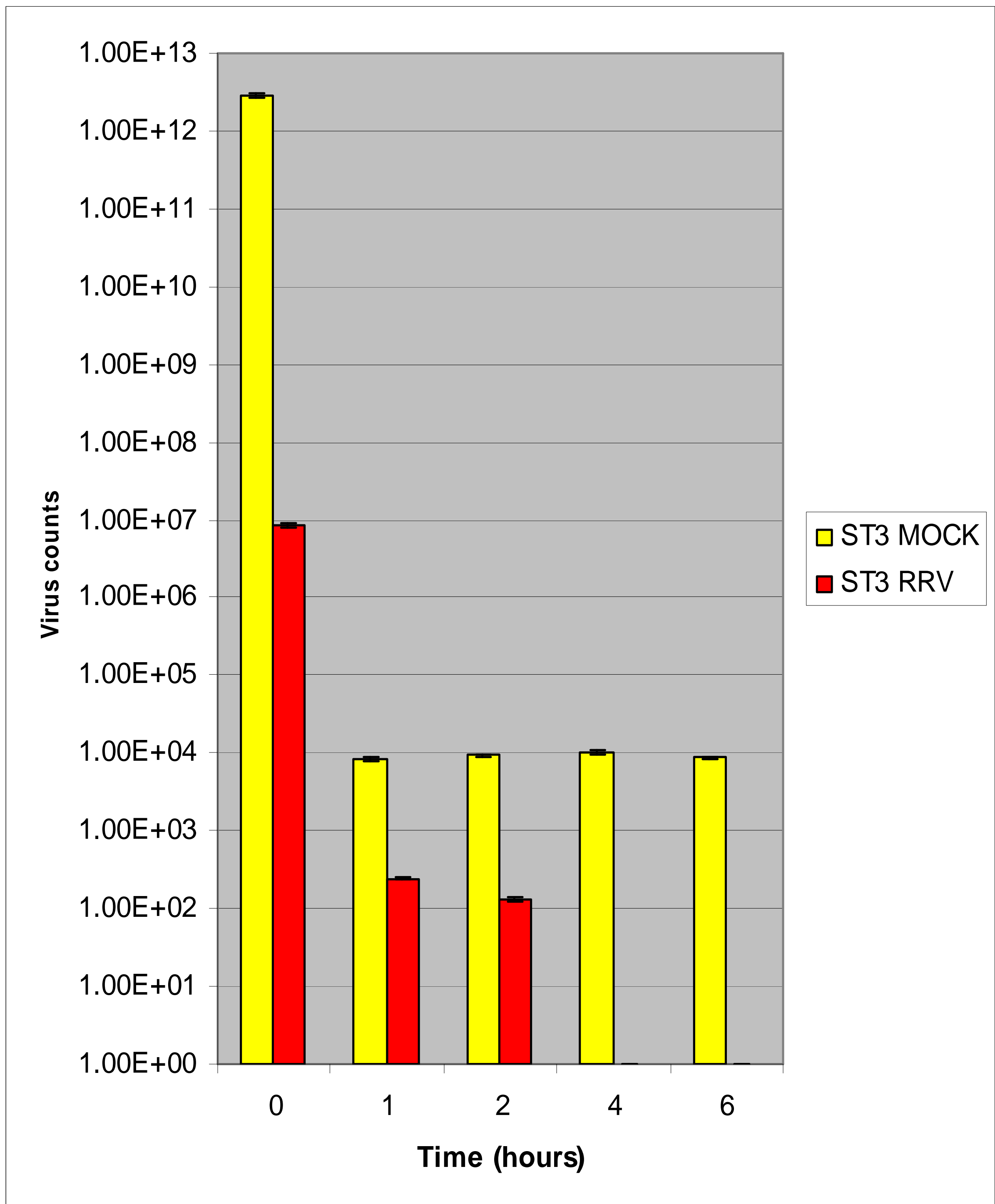

Figure 4.5 Reovirus ST3 (MRV-3DE) recovery from reovirus ST3 (MRV-3DE) superinfections.
(error bars represent 5% error)

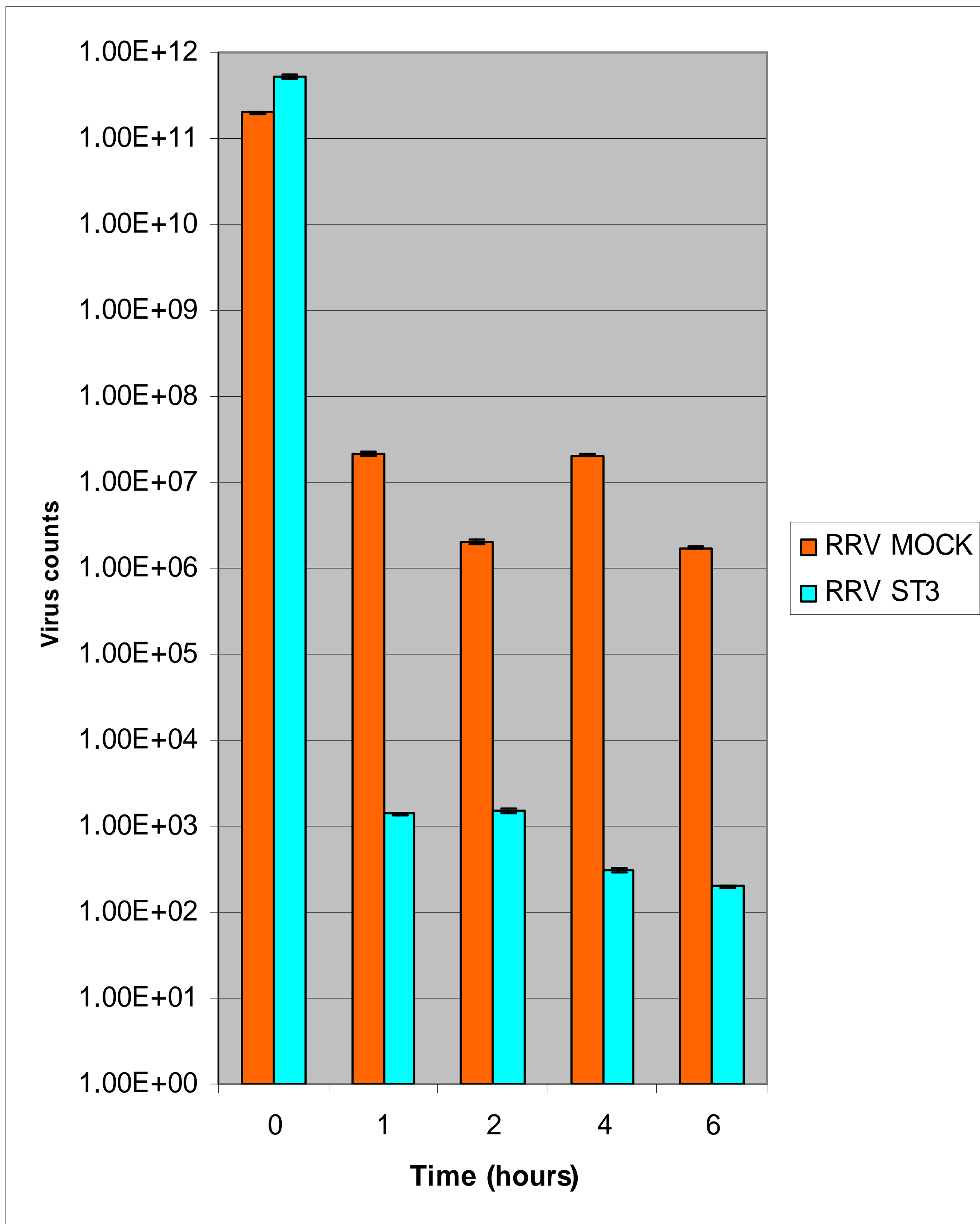

Figure 4.6 Rhesus rotavirus recovery from reovirus ST3 (MRV-3DE) superinfections. (error bars represent 5% error)

4.3 Rhesus Rotavirus and Reovirus Monoreassortant Co-infections

4.3.1 Rhesus Rotavirus and ST1wST3 Monoreassortants

To determine if an individual reovirus gene or genes were responsible for the inhibition, co-infections with RRV and a set of ST1wST3 monoreassortants were performed. The monoreassortant viruses containing the L1, L3, S1, S2, and S3 genes from MRV-3DE were severely inhibited by the presence of RRV during co-infections. RRV slightly inhibited the ST1wST3 monoreassortant containing the L2 gene. The ST1wST3 monoreassortants containing the MRV-3DE genes M1, M2, M3, and S4 genes were not affected by the presence of RRV in the co-infections (Figure 4.8 and Table A.13). T-tests revealed that the L1, L2, L3, S1, S2, and S3 monoreassortants were significantly inhibited by the presence of RRV (*p* values = 0.000) (Table A.14).

The same co-infections revealed that the monoreassortants containing the MRV-3DE genes L1, L3, M3, S1, S2, and S3 severely inhibited RRV infectious particle production five- to nine-fold during co-infections. The ST1wST3 monoreassortants containing the MRV-3DE genes L2, M1, M2, and S4 genes completely inhibited all RRV infectious particle production during co-infections (Figure 4.9 and Table A.15). Statistical analysis using t-tests indicated that nine of the ST1wST3 monoreassortants (L1, L2, L3, M1, M2, M3, S2, S3, and S4) significantly inhibited RRV production during co-infections (*p* values = 0.000, 0.005, 0.000, 0.000, 0.002, 0.002, 0.000, 0.000, 0.000, respectively) (Table A.16).

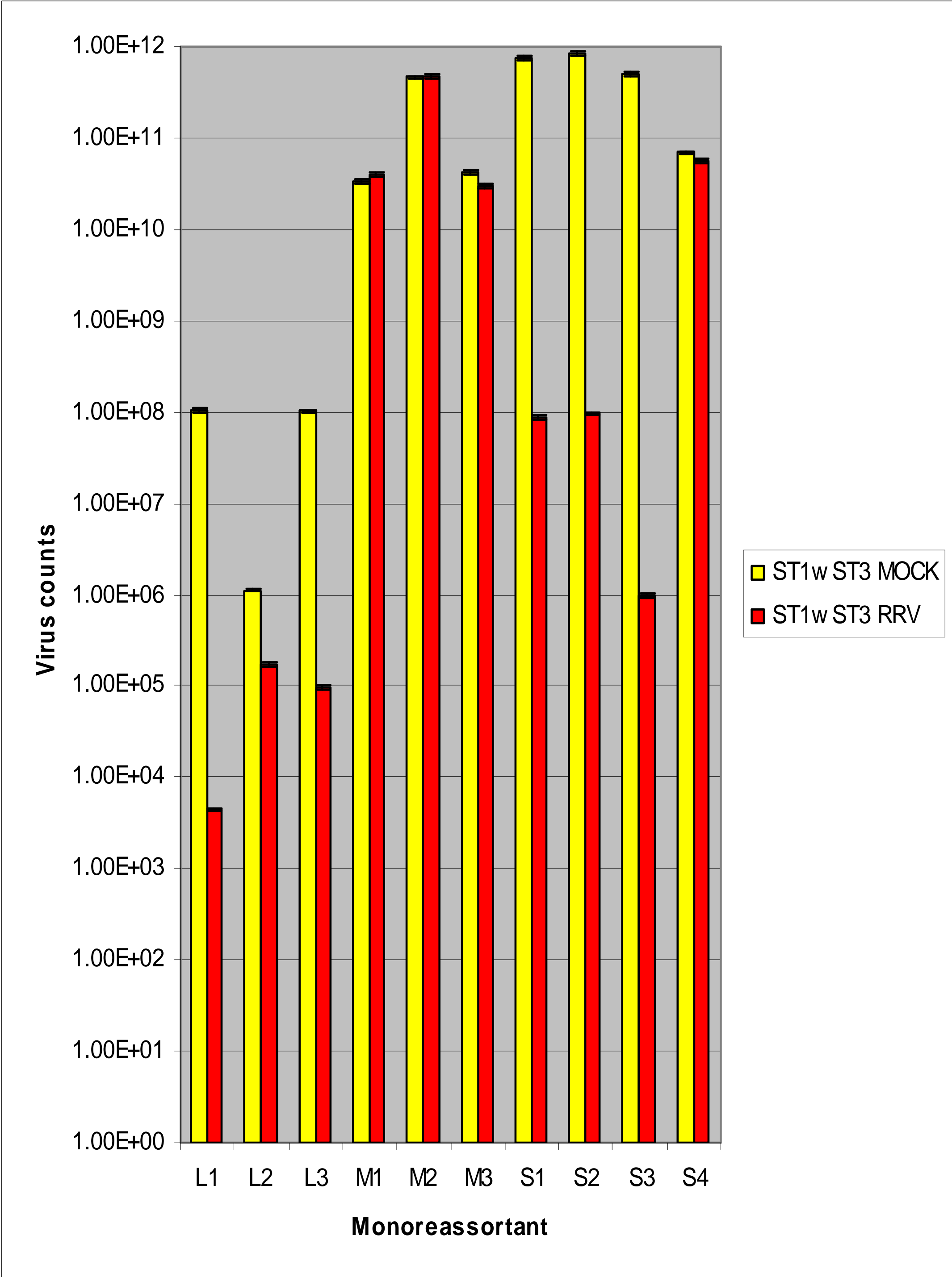

Figure 4.7 ST1wST3 recovery from RRV co-infections. (error bars represent 5% error)

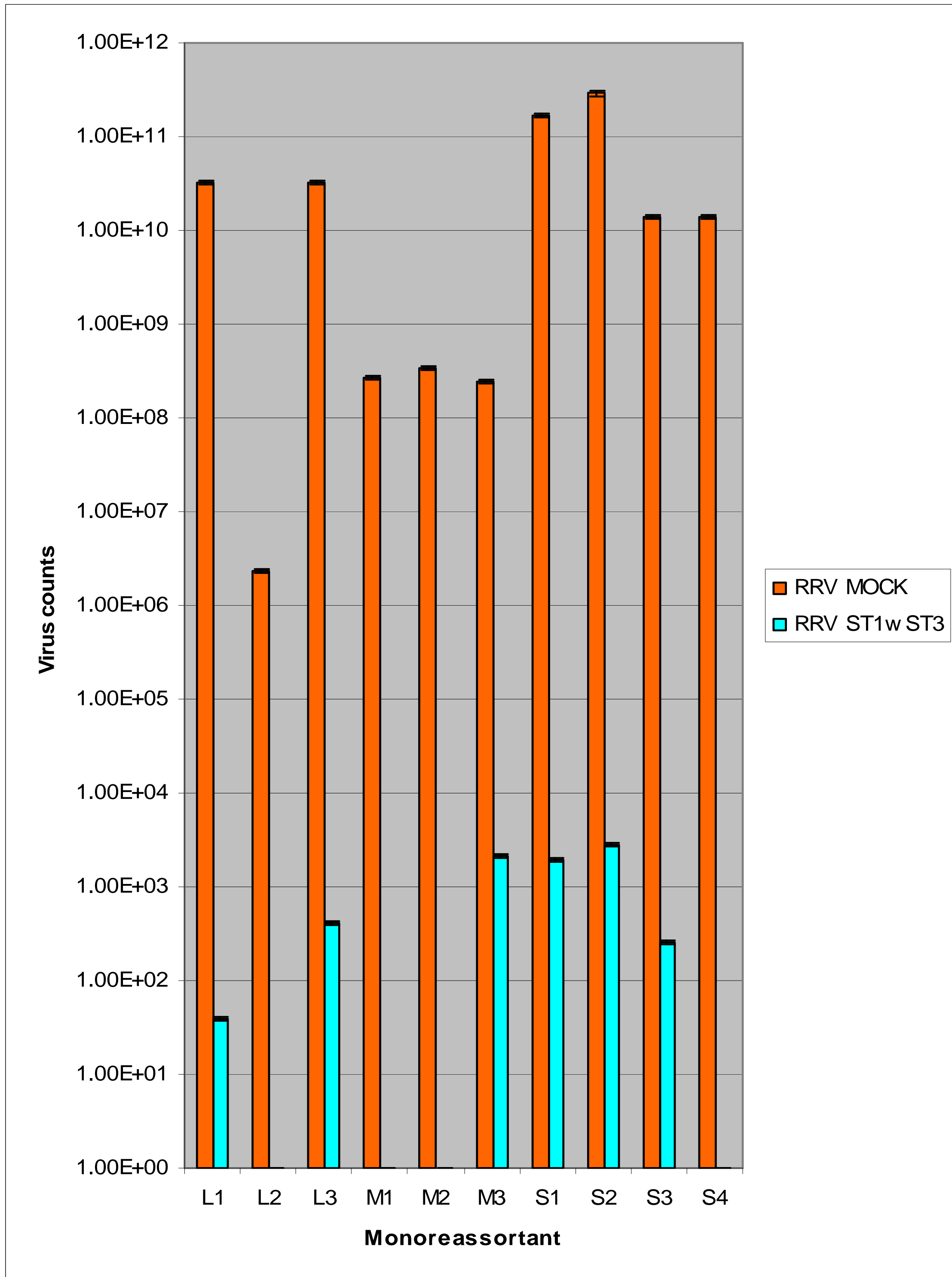

Figure 4.8 RRV recovery from ST1wST3 co-infections. (error bars represent 5% error)

4.3.2 Rhesus Rotavirus and ST3wST2 Monoreassortants

An additional set of monoreassortants, ST3wST2, was used to help determine which reovirus genes were responsible for the inhibition of RRV during co-infections. The monoreassortants containing MRV-2JO genes L3, S1, and S3 were moderately inhibited (one- to two-fold) by the presence of RRV during co-infections. The monoreassortants containing the L1 gene from MRV-2JO was slightly inhibited by the presence of RRV during co-infections. The monoreassortants containing the M2 and S2 genes of MRV-2JO origin were completely inhibited by the presence of RRV during co-infections. Finally, the monoreassortants containing the L2, L3, M1 and S4 genes from MRV-2JO were not negatively affected by the presence of RRV during co-infection. The L2 and S4 monoreassortants were not affected by RRV in the co-infections. Their productions during co-infections were comparable to their productions in single infections. The L3 monoreassortant production during co-infections was enhanced approximately one-fold in comparison to the single infections. The M1 monoreassortant was enhanced approximately two-fold by the presence of RRV during the co-infections in comparison to the single infections (Figure 4.10 and Table A.17). T-tests revealed significant results for infectious particle production for the MRV-2JO genes L1, M1, M3, S1, S2, and S3 (p values = 0.003, 0.001, 0.000, 0.001, 0.002, and 0.003, respectively) (Table A.18).

The same co-infections revealed that the monoreassortants containing the MRV-2JO genes L1, L2, L3, M1, M2, M3, S1, and S4 significantly inhibited RRV infectious virus production during co-infections between one- and almost four-fold. The monoreassortants containing the S2 and S3 genes of MRV-2JO origin completely inhibited RRV infectious particle production (Figure 4.11 and Table A.19). T-tests revealed that ST3wST2 monoreassortants significantly inhibited RRV production during co-infections when the L1, L3, M1, M2, M3, S1, S2, S3, and S4 MRV-2JO genes were present (p values = 0.000 - 0.001) (Table A.20).

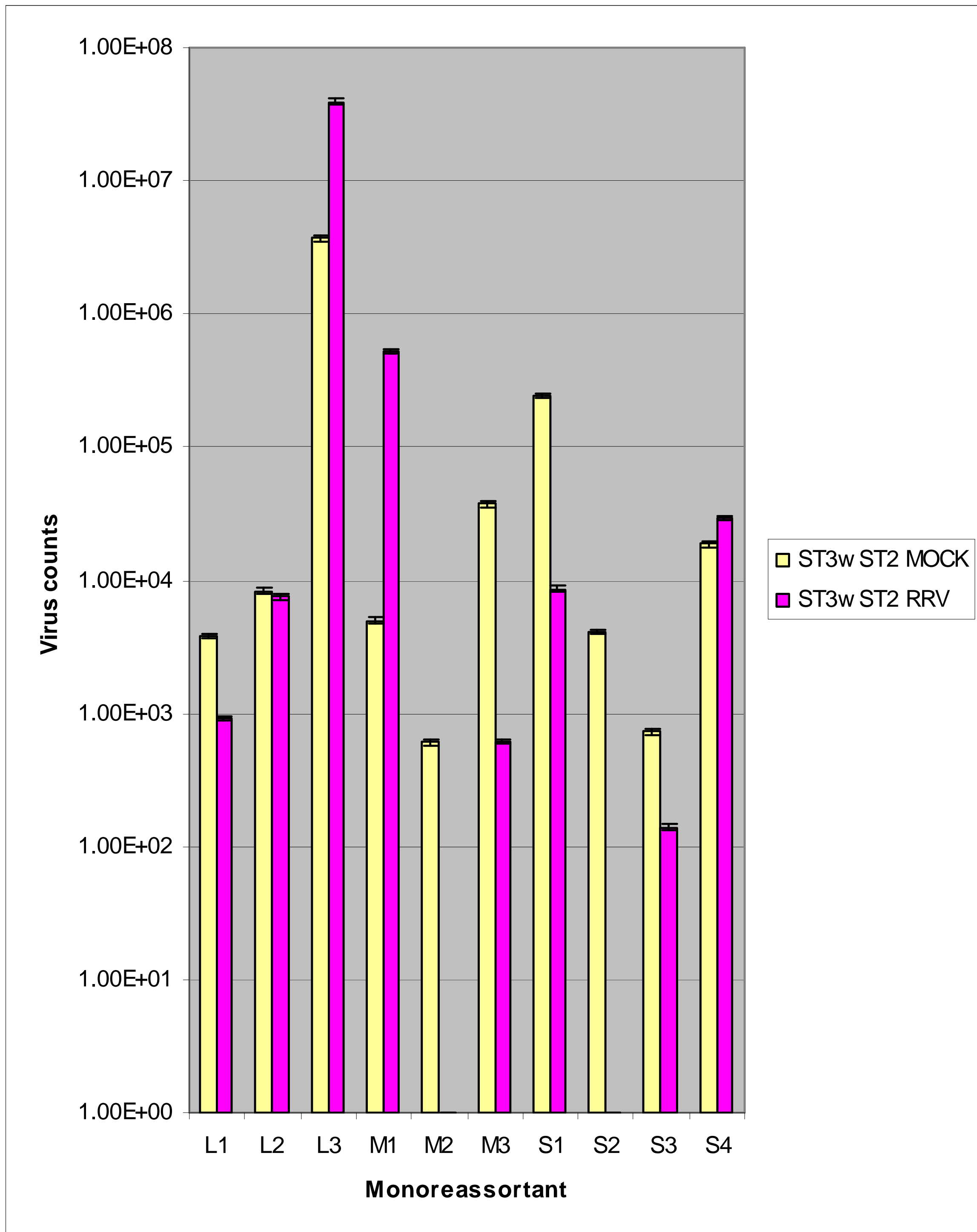

Figure 4.9 ST3wST2 recovery from RRV co-infections. (error bars represent 5% error)

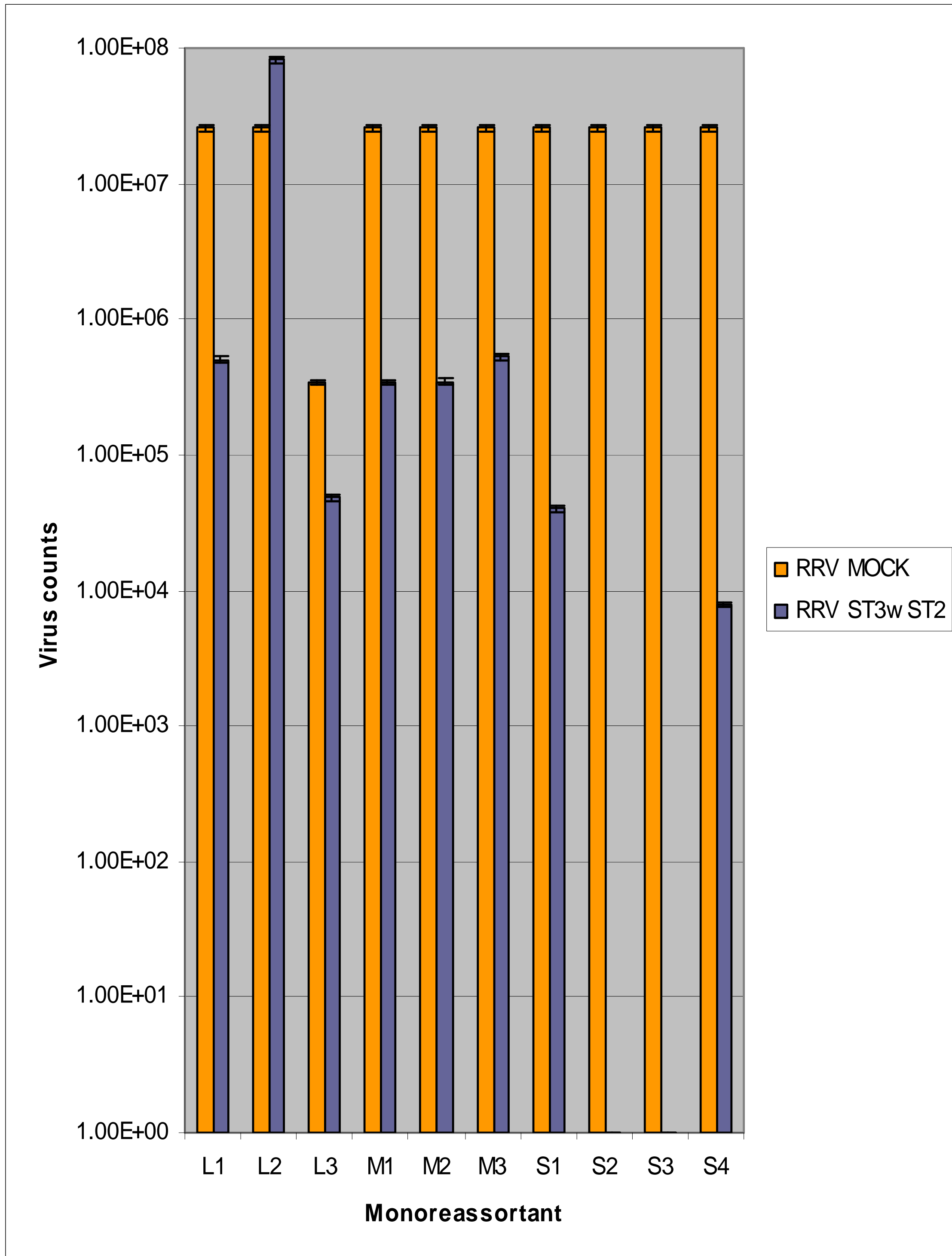

Figure 4.10 RRV recovery from ST3wST2 co-infections. (error bars represent 5% error)

4.4 Simian Rotavirus (SA11) and Reovirus MRV-1LA, MRV-2JO, and MRV-3DE Co-infections

To determine if reovirus had inhibitory actions against another strain of rotavirus, SA11, co-infections using SA11 and the three serotypes of reovirus were performed. SA11 had little effect on infectious particle production by MRV-1LA, MRV-2JO, and MRV-3DE. MRV-1LA and MRV-3DE were not affected by the presence of SA11 during co-infections. MRV-2JO was inhibited almost one-fold during co-infections with SA11 (Figure 4.12 and Table A.21). T-tests revealed that only MRV-2JO was significantly affected by SA11 during co-infections (p value = 0.005) (Table A.22).

These same co-infections revealed that all three serotypes of reovirus inhibited SA11 five- to seven-fold. T-tests revealed that MRV-1LA, MRV-2JO, and MRV-3DE significantly inhibited SA11 (p values = 0.001, 0.000, and 0.001, respectively) (Figure 4.13 and Tables A.23 and A.24).

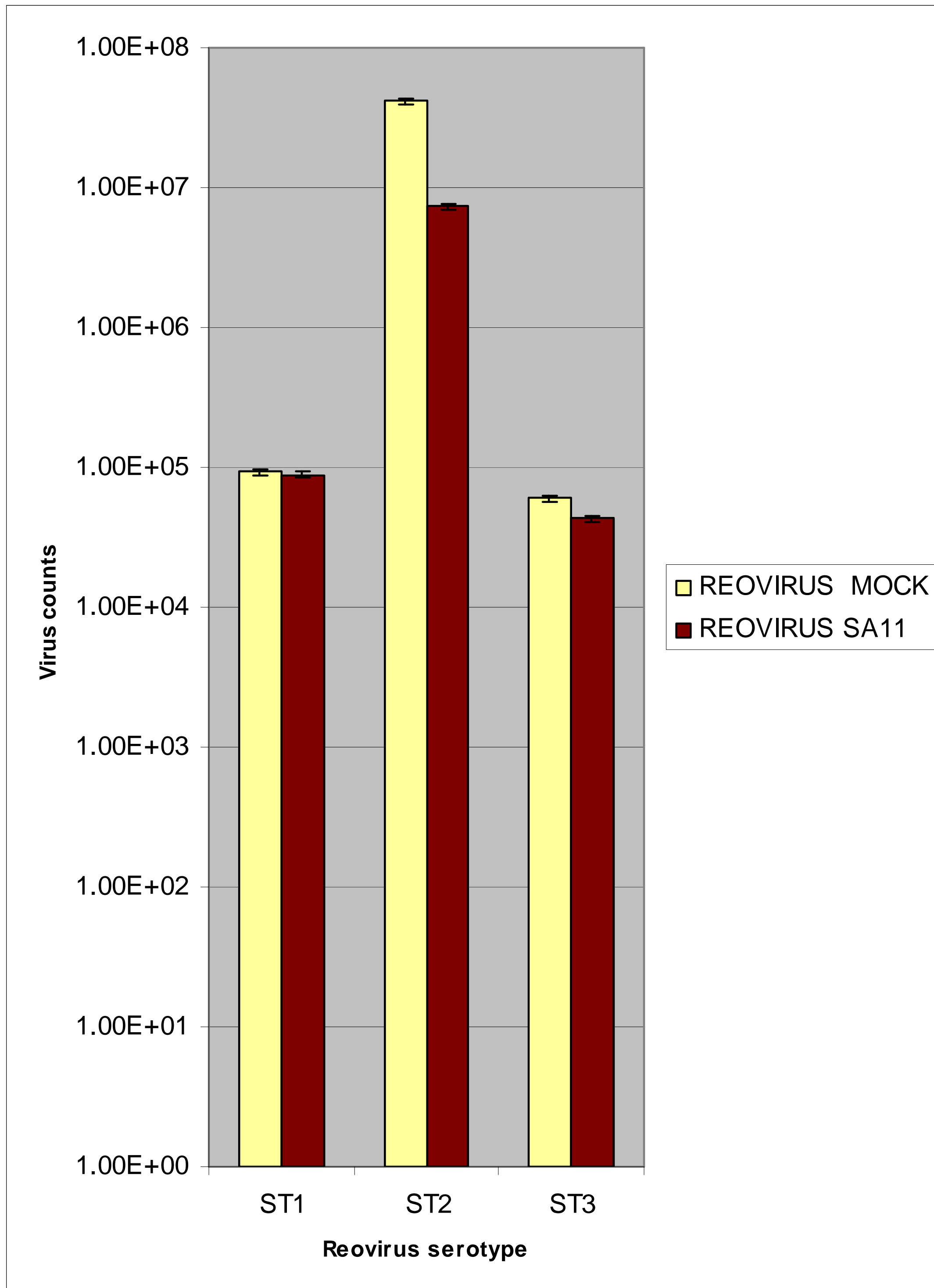

Figure 4.11 Reovirus recovery from SA11 co-infections. (error bars represent 5% error)

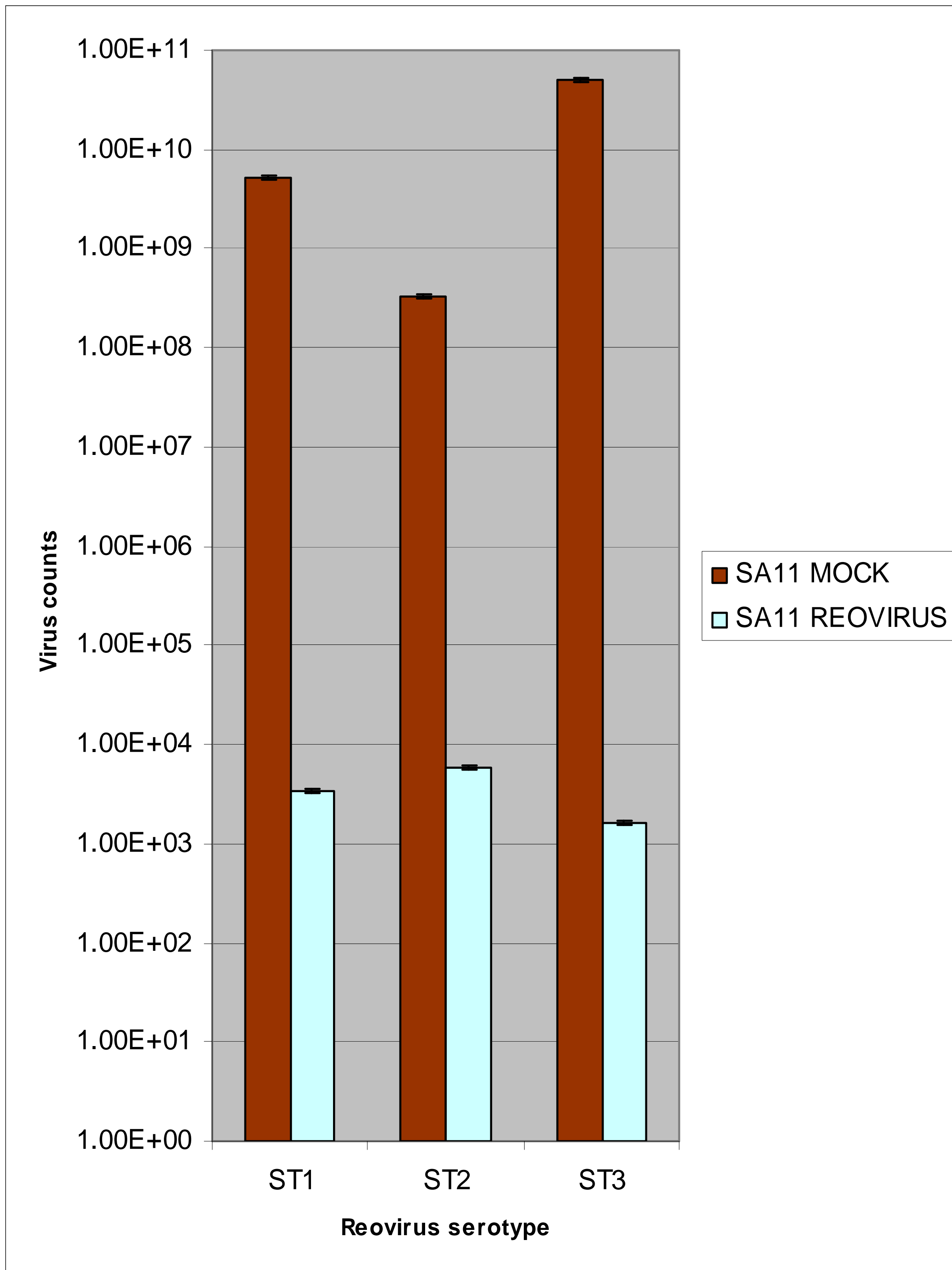

Figure 4.12 SA11 recovery from reovirus co-infections. (error bars represent 5% error)

4.5 *In Vivo* Co-infections

4.5.1 Analysis of the Impact of Co-infections on the Development of Diarrhea in Mice as a Model

The main reason for performing *in vivo* infections using mice as a model was to determine if reovirus exhibited the same inhibition of rotavirus production in mammals as it did in *in vitro* experiments. Mice were checked for diarrhea everyday for seven days by abdominal palpitations beginning on day one of treatments. When diarrhea developed in the mice, it always occurred on days two through six, with all but two instances occurring on days two through five. The MEM treatment and the MRV-1LA treatment did not result in any cases of diarrhea during the seven days. The RRV treatment resulted in 16%-79% of mice developing diarrhea during days two through six. The co-infection treatment of RRV and MRV-1LA resulted in only 6%-33% of the inoculated mice developing diarrhea during days four through six. This was a significant improvement over the percentage of diarrhea that developed in mice singularly inoculated with RRV. The treatment of RRV followed by MRV-1LA resulted in 37%-74% of the mice developing diarrhea on days three through five. Six to 38% of mice that received MRV-3DE only developed diarrhea on days two through five. Finally, 10%-67% of mice that received the co-infection treatment of RRV and MRV-3DE developed diarrhea on days two through six (Figure 4.14 and Table A.27). T-tests revealed that co-infections in mice of RRV and MRV-1LA significantly lowered the percentage of mice that developed diarrhea on days two, three, and four in comparison to mice infected singularly with RRV (p value = 0.001, 0.007, and 0.005, respectively). Mice that were given RRV on days one through three and MRV-1LA on days three through five, only had significantly lower rates of diarrhea on one out of seven days in comparison to RRV single inoculation or RRV and MRV-1LA co-infections given on days one through three (Table A.28). Mice given simultaneous inoculations of RRV and MRV-1LA only developed diarrhea on days four through six in comparison to mice inoculated with only RRV who developed diarrhea for days two through six. Our analysis revealed that simultaneous co-infections of RRV and MRV-1LA reduced the percentage of mice that developed diarrhea from

RRV better than any other treatment explored. There were no in between litter effects observed among the litters of mice used in the study.

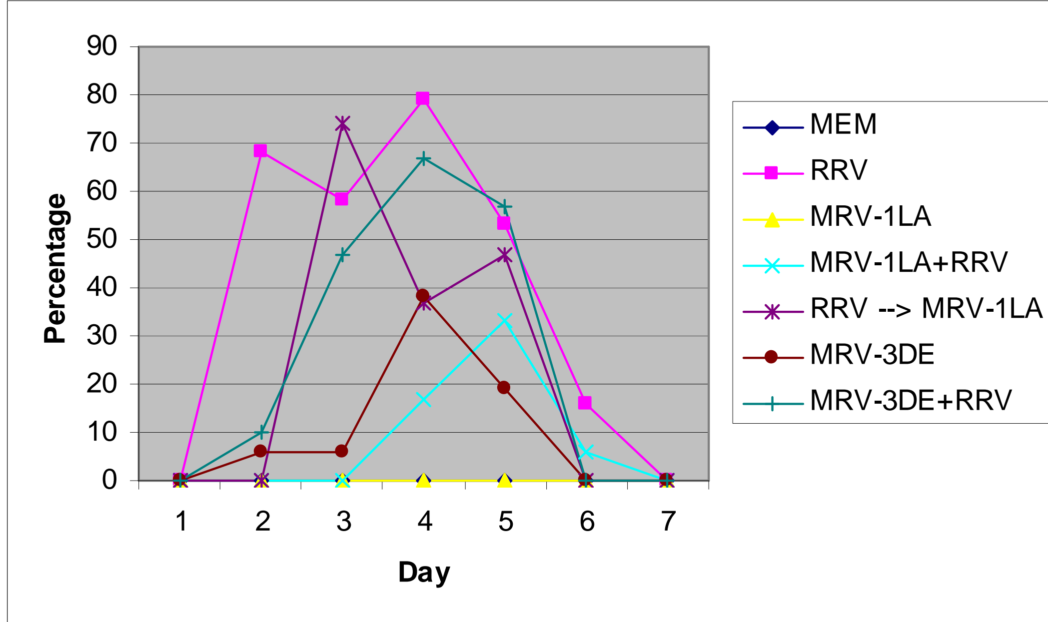

Figure 4.13 Percentage of mice that developed diarrhea from single and co-infections of rhesus rotavirus and reovirus

4.5.2 Analysis of the Impact of Co-infections In Vivo Using Growth Rates of Mice as a Measure

To address the possibility that co-infections had a broader impact on the health of the animals the growth of virus-infected mice was monitored for 60 days. *In vivo* experiments revealed no difference in growth over the course of two months regardless of the treatment the subjects were given. Groups of mice were given oral inoculations of treatments (MEM, single virus, or co-infections of two viruses) for three days beginning on day one (actually day six of life). Mice were weighed daily for seven days starting on day one. After the initial seven days, all mice were weighed once every seven days until day 56. The average weights for the three groups that received each treatment were analyzed. T-tests revealed no significant differences of mouse weights on days 7, 28, and 56 between MEM treatments and viral treatments (Figure 4.15 and Tables A.25 and A.26).

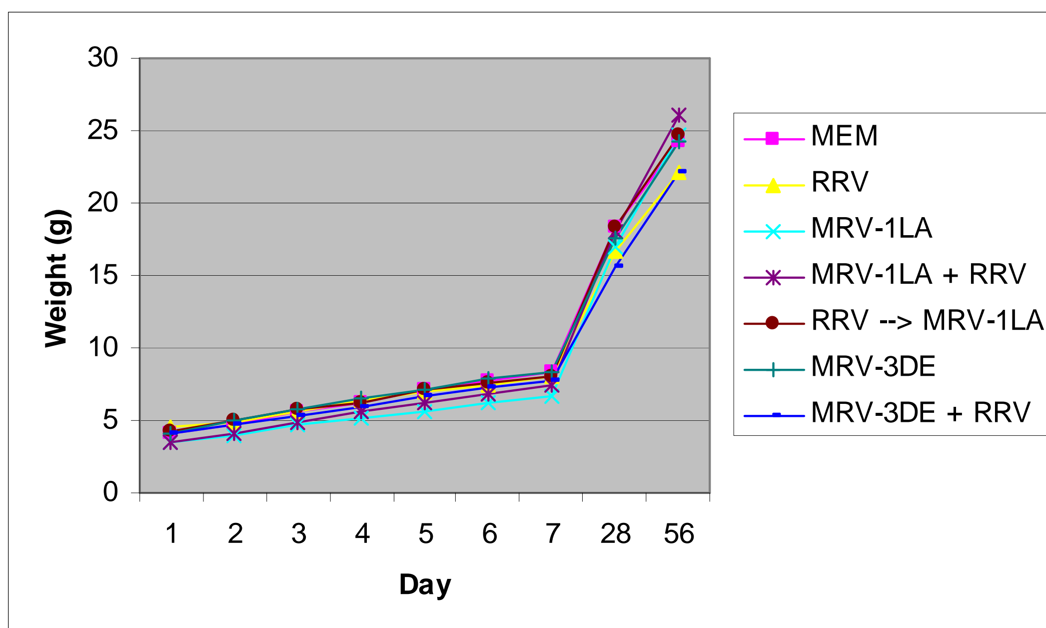

Figure 4.14 Average mouse weights for each treatment. (RRV → MRV-1LA indicates groups that received RRV for days 1-3 and MRV-1LA for days 3-5)

4.6 *In Vitro* dsRNA Analysis

An attempt was made to further characterize the co-infections by examining the genomic dsRNA produced during these infections (Figure 4.16). The total yield of progeny virus under these conditions is very low and attempts to sufficiently label individual genome segments from both viruses was not successful. The genome segments of all dsRNA viruses examined to date are produced in equal molar amounts which are directly proportional to the amount of infectious virus produced.[37] In conclusion based on our limited findings, it is very unlikely that an analysis of the dsRNA would have provided insight into the genes controlling the interactions between these viruses during co-infections.

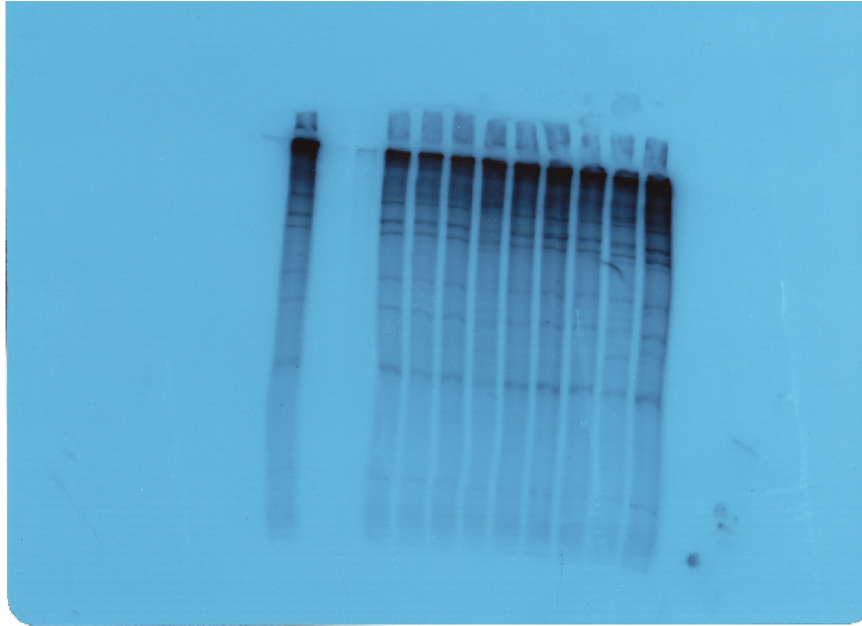

Figure 4.15 A sampling of dsRNA recovered from co-infections for analysis

4.7 *In Vitro* Protein Production Analysis

A logical extension of the work with the monoreassortants, was the examination of the levels of the individual proteins encoded by these genes. Analysis of the production of Reovirus proteins produced during single and co-infections was performed on the exposed developed films from the western blot procedure. The infections were performed with RRV and the L1, L3, S1, S2, and S3 ST1wST3 monoreassortants. The developed films revealed that the production of the reovirus large proteins (λ, the proteins produced by the large genome segments of the virus), medium proteins (μ, the proteins produced by the medium genome segments of the virus), and small proteins (σ, the proteins produced by the small genome segments of the virus) were affected in the co-infections when compared to the production of these same proteins in the single infections of the monoreassortants. In the co-infections, the L1 large protein production was increased 59%, the medium protein production was increased 48%, and the small production was decreased 7% (Figure 4.17 and Table 4.1). The L3 co-infections revealed the large protein production decreased 100%, the medium protein production decreased 64%,

and the small protein production decreased 23%. The S1 co-infections revealed a 100% decrease in large protein production, a 96% decrease in medium protein production, and a 73% decrease in small protein production. The S2 co-infections revealed a 100% decrease in large protein production, a 51% decrease in medium protein production, and a 65% small protein production decrease. Finally, the S3 co-infections revealed a 100% decrease in the production of the large proteins, a 90% decrease in the production of medium proteins, and an 86% decrease in the production of the small proteins.

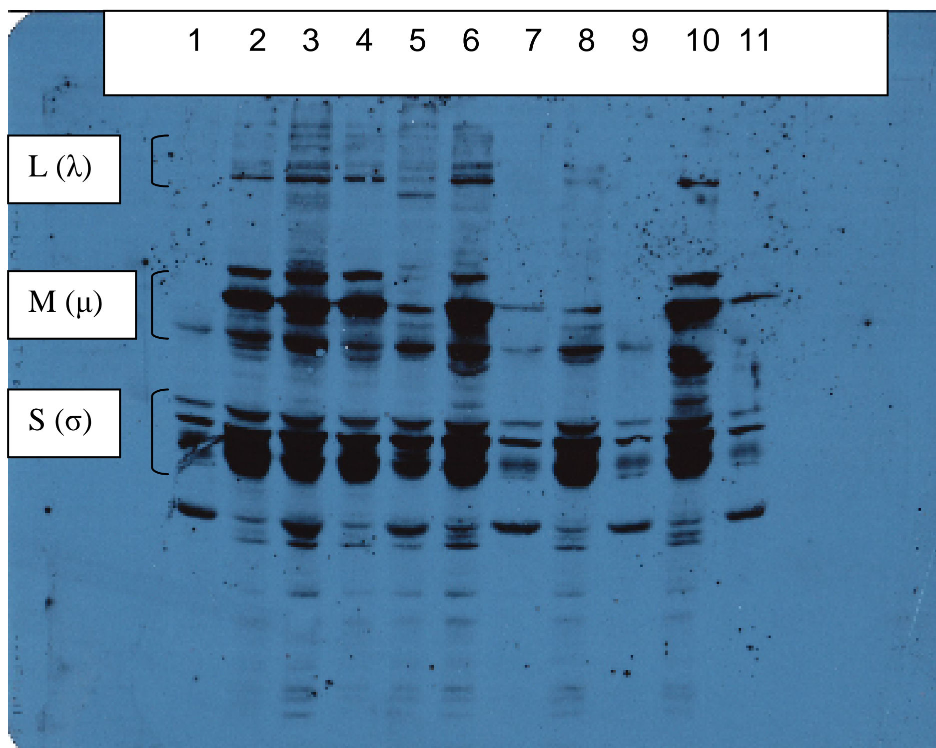

Figure 4.16 Reovirus proteins produced during single and co-infections (MOI=10) of L1, L2, S1, S2, and S3 ST1wST3 monoreassortants and RRV. (Lanes: 1-RRV control, 2-L1, 3-RRV&L1, 4-L3, 5-RRV&L3, 6-S1, 7-RRV&S1, 8-S2, 9-RRV&S2, 10-S3, 11-RRV&S3)

Table 4.1 Ratio of proteins produced in co-infections in comparison to single infections

Infection	**Amount of Protein**		
	L (λ)	**M (μ)**	**S (σ)**
MR-L1	1	1	1
MR-L1 & RRV	1.6	1.5	0.9
MR-L3	1	1	1
MR-L3 & RRV	0	0.4	0.8
MR-S1	1	1	1
MR-S1 & RRV	0	0	0.3
MR-S2	1	1	1
MR-S2 & RRV	0	0.5	0.4
MR-S3	1	1	1
MR-S3 & RRV	0	0.1	0.1

These protein production results follow the pattern of progeny virus produced by the monoreassortants during *in vitro* co-infections with RRV. There is a decrease in all of the monoreassortants' protein production for all three protein classes, except for the L1 monoreassortant. This corresponds to a significant decrease in the amount of infectious particle production for the L3, S1, S2 and S3 monoreassortants during the co-infections in comparison to their respective single infection production of progeny virus. The L1 monoreassortant actually had an increase in the production of the λ and μ proteins by about one and a half and a stable production of σ proteins. This increase of proteins could not be deduced from the amount of progeny infectious virus produced by the L1 monoreassortant during the co-infections because there was a significant amount of inhibition by RRV and not an increase in viral count. It is interesting to note that these monoreassortants allowed RRV not to be completely inhibited during co-infections, unlike the rest of the monoreassortant set. Possibly, the decreased production of the three classes of reovirus proteins lessened the inhibitory action of reovirus during the co-infections.

CHAPTER 5

DISCUSSION

Rotavirus infections are the number one cause of childhood diarrhea in the world. These infections result in hundreds of thousands of deaths in children under the age of five worldwide every year. There is no known cure for rotavirus, only costly preventative vaccinations that are not likely to be used in developing countries. The only treatment is currently oral rehydration therapy. New treatment options should be sought, other than just rehydration therapy. These new options could include preventative measures to try and prevent infection from occurring or measures to lessen the severity and duration of the infection once it occurs. These options would provide greater relief to those suffering from rotaviral infection as opposed to only treating the main symptom, dehydration of infection. Having multiple treatment options could greatly increase the quality of life and lifespan of young children worldwide. We feel that the work we have performed could lead to new treatment options for rotavirus infections. Determining which reovirus proteins are responsible for the inhibition of rotavirus production could lead to an effective treatment option. For example, an attenuated version of reovirus that contains the functional gene(s) that produces the inhibitory proteins but is not harmful to humans could be added to the water supply in high risk areas. This attenuated virus could be given as a prophylactic to at-risk individuals when a known rotavirus outbreak occurs.

My research to date has shown several positive results. Initial co-infections of MA-104 cells with rhesus rotavirus (RRV) and the three known human reovirus serotypes (MRV-1LA, MRV-2JO, and MRV-3DE) resulted in inhibition of rotavirus by MRV-1LA and MRV-2JO. When MA-104 cells were co-infected with RRV and MRV-1LA at the same time, MRV-1LA significantly inhibited production of infectious RRV particle production by three-fold. MRV-2JO had a more drastic effect on RRV production during co-infections. When MA104 cells were simultaneously

infected with MRV-2JO and RRV, MRV-2JO inhibited production of infectious RRV particle production by about eight-fold. MRV-3DE did not have any adverse effects on RRV during co-infections in MA104 cells. These results indicate that a gene or genes present in MRV-1LA and MRV-2JO, when present, can have a substantial impact on the production of infectious RRV particles during co-infections. These are significant discoveries considering that both RRV and reovirus are ubiquitous in nature and most children have antibodies to both viruses by the age of five. Co-infections of these two viruses are likely to occur in nature but have not been studied in depth to date.

Using these results, I tried to determine which specific gene or genes in MRV-1LA and MRV-2JO was responsible for the inhibition of RRV during co-infections. I performed separate co-infections with RRV and two sets of mammalian orthoreovirus monoreassortants. Reovirus monoreassortants are viruses that each contain one gene segment from one parental serotype (MRV-1LA, MRV-2JO, or MRV-3DE) and the remaining nine gene segments from another parental serotype. Historically, these sets of monoreassortants have been very useful in identifying the proteins responsible for several stages of the reovirus replication cycle. The two sets of monoreassortants used were MRV-1LA with MRV-3DE genes (ST1wST3) and MRV-3DE with MRV-2JO genes (ST3wST2). Each set includes ten viruses that each contains nine gene segments from one reovirus serotype and one gene segment from another reovirus serotype. The RRV and ST1wST3 co-infections performed in MA-104 cells resulted in complete inhibition of RRV by the L2, M1, M2, and S4 ST1wST3 monoreassortants. These four ST1wST3 monoreassortants prevented any detectable production of infectious RRV particle production during *in vitro* co-infections. These results narrow down the MRV-1LA genes potentially responsible for the RRV inhibition to the L1, L3, M3, S1, S2, and S3 genes. When these MRV-1LA genes are present during the co-infections with RRV, production of infectious RRV particles is completely inhibited below acceptable detection limits (i.e. <30 plaques).

The next set of co-infections using RRV and the ST3wST2 monoreassortants resulted in complete inhibition of infectious RRV particles when the S2 or S3 genes from MRV-2JO were present. The remaining eight ST3wST2 monoreassortants resulted in only mild to moderate RRV inhibition. These results narrow down the genes in common between MRV-1LA and MRV-2JO that completely inhibit infectious RRV particle production to the S2 and S3 genes.

The S2 and S3 genes play vital roles in the reovirus replication cycles. It is known that the S2 gene product, σ2 protein, is capable of binding dsRNA.[38] The S3 gene product, σNS protein, is vital for the synthesis of dsRNA and it plays a role in gene segment assortment.[39] The dsRNA and protein sequence identities of these two genes and proteins has been identified (Table 5.1).

Table 5.1 S2 and S3 gene and protein product sequence identities[44]

Gene or Protein Identity	**T1L:T2J**	**T2J:T3D**	**T1L:T3D**
S2 gene	77.5%	77.6%	85.8%
σ2 protein	94.3%	94.0%	98.8%
S3 gene	72.5%	73.6%	86.6%
σNS protein	86.1%	86.3%	97.3%

Since MRV-3DE had no impact on the production of infectious RRV particles when MA-104 cells were simultaneously infected with both viruses, the impact of superinfection was explored. This series of infections was examined to determine if giving one of the viruses a time advantage would effect the production of either virus' infectious particles. This phenomenon was explored because co-infections that occur in nature are more likely to be the product of superinfections and not co-infections that are initiated at the exact same time.

When cells were simultaneously infected with RRV and MRV-3DE, there was no inhibition of RRV production and a six-fold decrease in MRV-3DE production. When cells already infected with MRV-3DE were infected with RRV between one and six hours later,

infectious RRV particle production was inhibited about seven-fold and infectious MRV-3DE production was inhibited about two-fold. It did not matter if the superinfection occurred one, two, four, or six hours after the initial MRV-3DE infection, the level of inhibition of both viruses was the same. It appears that giving MRV-3DE even a one hour advantage in the cells before RRV was added, allowed it to overcome some of the inhibition caused by RRV during co-infections. Conversely, the time advantage given to MRV-3DE allowed the level of inhibition of infectious RRV particle production to go from zero to seven-fold. It suggests that the inhibition of RRV is at the level of entry into the cells.

In the next set of superinfections I looked at MRV-3DE superinfection to determine if a time advantage of RRV in the infections would lessen the level of inhibition caused by MRV-3DE. When RRV was in the cells for one to six hours before the MRV-3DE superinfection, the level of inhibition on RRV particle production was only four-fold. This is a three-fold decrease in inhibition on RRV particle production when compared to the RRV superinfections. So, even the one hour time advantage given to RRV allowed it to overcome a great deal of the inhibition caused by MRV-3DE. On the other hand, when RRV was in cells for four to six hours before MRV-3DE superinfection, a complete inhibitor of infectious MRV-3DE particle production was observed. When RRV was in cells for one to two hours before MRV-3DE superinfection occurred, only a two-fold decrease in infectious MRV-3DE particle production occurred. Overall, it was determined that if RRV infection occurs simultaneously with MRV-3DE, then no inhibition of RRV production occurs. When RRV superinfection occurs in cells already infected with MRV-3DE, then a great deal of inhibition of infectious particle production was seen. Finally, when cells already infected with RRV underwent an MRV-3DE superinfection, infectious RRV particle production was only moderately inhibited.

The final *in vitro* work looked at co-infections with SA11 and the three reovirus serotypes. This was done to determine if the level of inhibition seen with RRV carried over to another strain of rotavirus, SA11. MA-104 cells were simultaneously infected with SA11 and

MRV-1LA, MRV-2JO, or MRV-3DE. All three reovirus serotypes inhibited SA11 by about seven-fold during the co-infections. SA11 did not have any noticeable affect on infectious reovirus production regardless of the serotype. These results indicate that reovirus has the ability to inhibit more than one strain of rotavirus.

After determining that MRV-1LA and MRV-2JO successfully inhibited infectious RRV particle production *in vitro*, I decided to perform *in vivo* infections. Mice were used as the mammal model to determine if the RRV inhibitions seen in MA-104 cells during *in vitro* co-infections would carry over into living mammals. Six day old mice housed in micro-isolator cages (to prevent them from exposure to outside pathogens) were orally inoculated with single virus (RRV, MRV-1LA, MRV-2JO, or MRV-3DE) or two viruses (RRV+MRV-1LA, RRV+MRV-2JO, or RRV+MRV-3DE). The mice were inoculated daily for three days to ensure that they were exposed to the viruses enough to establish infection. Beginning on the first day of inoculation, all mice were weighed and checked for diarrhea by abdominal palpitations for a total of seven days. After seven days, all mice were checked only every seven days for weight and diarrhea for a total of 56 days. This was to ensure that the viruses caused no lasting detrimental effects on the mice.

At the end of the seven days, I determined that RRV successfully and consistently caused diarrhea in the mice. The mice infected with MRV-1LA and MRV-3DE did not develop diarrhea. The first group infected with MRV-2JO completely died within three days of the initial inoculation, this is most likely due to the fact that MRV-2JO is the most neurotropic of the three serotypes. These mice had no signs of diarrhea when they died so I assume that the neurotropic aspect of MRV-2JO is the cause of death. MRV-2JO was not used in further *in vivo* infections because of its high mortality rate.

Since establishing that RRV did consistently cause diarrhea and MRV-1LA and MRV-3DE did not, co-infections were performed to determine if the presence of one of the reovirus serotypes would inhibit or lessen the diarrhea caused by RRV in mice. Mice co-infected with

RRV and MRV-3DE did not have a lessened degree of diarrhea over the seven days in comparison to mice singularly infected with RRV. Mice that were co-infected with RRV and MRV-1LA did develop significantly less diarrhea than mice infected with RRV alone. These results indicated that the presence of MRV-1LA had a negative effect on the production of RRV *in vivo*. This resulted in a lessened severity of diarrhea to develop in the mice.

Recognizing that co-infections in nature will not always occur at the same time, I performed co-infections in mice where the mice were infected with RRV and then superinfected with MRV-1LA. This was done to determine if MRV-1LA superinfection could lessen the diarrhea already developed from RRV infections. Mice were inoculated with RRV on days one through three of treatment and they consistently developed diarrhea, as expected. Beginning on day three, all mice were inoculated with MRV-1LA for three days (days three to five of treatment). These mice did not have a significantly lessened severity or shortened duration of diarrhea than mice co-infected with both viruses simultaneously.

These results indicated that the best inhibition of RRV *in vivo* is when the subject is inoculated with RRV and MRV-1LA at the same time. Since it is impossible to know exactly when an individual will be infected with rotavirus it would not be known when to inoculate the individual with MRV-1LA to prevent possible life-threatening diarrhea. The best option would be to add an attenuated form of MRV-1LA (unable to cause harmful infections in individuals but still able to produce the inhibitory S2 and S3 proteins) to the water supply as a prophylactic to prevent rotaviral diarrhea if rotavirus infection was established. Another option is to use the attenuated MRV-1LA virus only when a known rotavirus outbreak is occurring. This attenuated MRV-1LA could be put in special bottles of water or electrolyte fluids, for example, and given to children in at-risk areas before they show signs of diarrhea from rotavirus. This could protect them from developing potentially life threatening rotaviral diarrhea.

With all the *in vitro* and *in vivo* work completed, I tried to determine at what level the inhibition of RRV occurs. MA-104 cells were singularly and dual infected with RRV and

ST1wST3 reovirus monoreassortants. This was done to determine if all RRV genes were replicated equally or if there was a decrease in the amount of certain RRV genes produced. Even though usable data was not obtained during our process, it is suspected that there is an overall reduction in dsRNA production of RRV, not a reduction in individual genome segments.[37]

The final aspect of this project was the evaluation of reovirus protein production during co-infections. MA-104 cells were co-infected with equal amounts of RRV and certain ST1wST3 reovirus monoreassortants. The L1, L3, S1, S2, and S3 ST1wST3 monoreassortants were used for the co-infections because those monoreassortants were the most inhibited during co-infections with RRV in the *in vitro* infections. The L1 monoreassortant was the only one that actually had an increase in the amount of the λ and μ proteins and only a slight decrease in the production of σ proteins. All of the other monoreassortants (L3, S1, S2 and S3) studied in the protein analysis had a significant decrease in the amounts of all three classes of proteins produced by reovirus. These results indicated that RRV was able to inhibit protein production, either directly or by inhibiting the production of their corresponding genome segments. Unfortunately, we could not analyze the amounts of proteins produced by RRV during the co-infections since RRV antibodies for the western blot analysis were not readily available after an exhaustive search.

My results could potentially offer new treatment options for an unnecessarily fatal illness. The next step would be to pursue more in depth animal testing with the co-infections. Once that was completed, producing an attenuated version of the MRV-1LA virus should be performed. This new virus could be used to determine its effectiveness in inhibiting RRV infectious particle production in *in vitro* infections and then in *in vivo* infections. It is extremely promising to have identified a new potential preventative treatment for such a devastating illness that affects so many children worldwide.

APPENDIX A

IN VITRO AND *IN VIVO* CO-INFECTIONS

Table A.1. Reovirus MRV-1LA, MRV-2JO, and MRV-3DE Infectious Particle Count from Rhesus Rotavirus Co-Infections

Reovirus Serotype	Reovirus Single Infection Virus Count	Reovirus Co-Infection Virus Count
MRV-1LA	9.81×10^{12}	8.53×10^{14}
MRV-2JO	7.93×10^{7}	7.96×10^{7}
MRV-3DE	2.81×10^{12}	8.63×10^{6}

Table A.2. Analysis of MRV-1LA, MRV-2JO, and MRV-3DE Counts in Co-Infections

Reovirus Serotype	t Value	p Value	Degrees of Freedom
MRV-1LA	8.950	0.003	3
MRV-2JO	0.059	0.956	3
MRV-3DE	-20.125	0.000	3

Table A.3. Rhesus Rotavirus Infectious Particle Count from Reovirus Co-Infections

Co-Infection Type (Reovirus Serotype)	RRV Single Infection Virus Count	RRV Co-Infection Virus Count
MRV-1LA	1.53×10^{9}	4.08×10^{6}
MRV-2JO	4.58×10^{9}	2.49×10^{2}
MRV-3DE	1.98×10^{11}	5.31×10^{11}

Table A.4. Analysis of Rhesus Rotavirus Counts in Reovirus Co-Infections

Co-Infection Type (Reovirus Serotype)	t Value	p Value	Degrees of Freedom
MRV-1LA	-27.256	0.000	3
MRV-2JO	-27.280	0.000	3
MRV-3DE	-1.115	0.346	3

Table A.5. MRV-3DE Infectious Particle Count from Rhesus Rotavirus Superinfections

Time MRV-3DE Was in Cells Before RRV Addition	MRV-3DE Single Infection Virus Count	MRV-3DE Co-Infection Virus Count
1 hours	4.44×10^{6}	3.43×10^{4}
2 hours	1.48×10^{6}	4.10×10^{4}
4 hours	1.54×10^{6}	4.63×10^{4}
6 hours	2.36×10^{6}	3.26×10^{4}

Table A.6. Analysis of MRV-3DE Counts in Rhesus Rotavirus Superinfections

Time MRV-3DE Was In Cells Before RRV Addition	t Value	p Value	Degrees of Freedom
1 hours	-24.148	0.000	3
2 hours	-20.179	0.000	3
4 hours	-13.317	0.000	3
6 hours	-85.887	0.000	3

Table A.7. Rhesus Rotavirus Infectious Particle Count from Rhesus Rotavirus Superinfections

Time MRV-3DE Was In Cells Before RRV Addition	RRV Single Infection Virus Counts	RRV Co-Infection Virus Counts
1 hours	$9.00x10^{9}$	$2.25x10^{3}$
2 hours	$1.28x10^{10}$	$1.59x10^{4}$
4 hours	$2.10x10^{9}$	$2.80x10^{3}$
6 hours	$1.88x10^{9}$	$1.86x10^{3}$

Table A.8. Analysis of Rhesus Rotavirus Counts in Rhesus Rotavirus Superinfections

Time MRV-3DE Was In Cells Before RRV Addition	t Value	p Value	Degrees of Freedom
1 hours	-48.389	0.000	3
2 hours	-11.449	0.001	3
4 hours	-18.783	0.000	3
6 hours	-5.045	0.000	3

Table A.9. MRV-3DE Infectious Particle Count from MRV-3DE Superinfections

Time RRV Was In Cells Before MRV-3DE Addition	MRV-3DE Single Infection Virus Counts	MRV-3DE Co-Infection Virus Counts
1 hours	$8.25x10^{3}$	$2.43x10^{2}$
2 hours	$9.36x10^{3}$	$1.30x10^{2}$
4 hours	$1.02x10^{4}$	<30
6 hours	$8.66x10^{3}$	<30

Table A.10. Analysis of MRV-3DE Counts in MRV-3DE Superinfections

Time RRV Was In Cells Before MRV-3DE Addition	t Value	p Value	Degrees of Freedom
1 hours	-55.549	0.000	3
2 hours	-38.527	0.000	3
4 hours	-91.375	0.000	3
6 hours	-8.003	0.004	3

Table A.11. Rhesus Rotavirus Infectious Particle Count from MRV-3DE Superinfections

Time RRV Was In Cells Before MRV-3DE Addition	RRV Single Infection Virus Counts	RRV Co-Infection Virus Counts
1 hours	$2.18x10^{7}$	$1.39x10^{3}$
2 hours	$2.06x10^{6}$	$1.50x10^{3}$
4 hours	$2.08x10^{7}$	$3.10x10^{2}$
6 hours	$1.74x10^{6}$	$1.99x10^{2}$

Table A.12. Analysis of Rhesus Rotavirus Counts in MRV-3DE Superinfections

Time RRV Was In Cells Before MRV-3DE Addition	t Value	p Value	Degrees of Freedom
1 hours	-11.011	0.002	3
2 hours	-11.449	0.001	3
4 hours	-6.490	0.007	3
6 hours	-17.340	0.000	3

Table A.13. ST1wST3 Monoreassortant Infectious Particle Count from Rhesus Rotavirus Co-Infections

ST1wST3 Monoreassortant (MRV-3DE Gene)	ST1wST3 Monoreassortant Single Infection Virus Counts	ST1wST3 Monoreassortant Co-Infection Virus Counts
L1	$1.05x10^{8}$	$4.45x10^{3}$
L2	$1.12x10^{6}$	$1.70x10^{5}$
L3	$1.01x10^{8}$	$9.81x10^{4}$
M1	$3.32x10^{10}$	$4.00x10^{10}$
M2	$4.61x10^{11}$	$4.68x10^{11}$
M3	$4.28x10^{10}$	$2.93x10^{10}$
S1	$7.41x10^{11}$	$8.60x10^{7}$
S2	$8.29x10^{11}$	$9.55x10^{7}$
S3	$4.99x10^{11}$	$9.78x10^{5}$
S4	$6.88x10^{10}$	$5.49x10^{10}$

Table A.14. Analysis of ST1wST3 Monoreassortant Virus Counts in Rhesus Rotavirus Co-Infections

ST1wST3 Monoreassortant	t Value	p Value	Degrees of Freedom
L1	-28.173	0.000	3
L2	-32.857	0.000	3
L3	-75.356	0.000	3
M1	1.995	0.140	3
M2	0.091	0.933	3
M3	-2.755	0.070	3
S1	-17.139	0.000	3
S2	-49.396	0.000	3
S3	-44.632	0.000	3
S4	-2.785	0.069	3

Table A.15. Rhesus Rotavirus Infectious Particle Count from ST1wST3 Monoreassortant Co-Infections

Co-Infection Type: ST1wST3 Monoreassortant (MRV-3DE Gene)	RRV Single Infection Virus Counts	RRV Co-Infection Virus Counts
L1	3.26×10^{10}	3.88x10
L2	2.29×10^{6}	<30
L3	3.26×10^{10}	4.03×10^{2}
M1	2.68×10^{8}	<30
M2	3.39×10^{8}	<30
M3	2.48×10^{8}	2.09×10^{3}
S1	1.67×10^{11}	1.94×10^{3}
S2	2.89×10^{11}	2.81×10^{3}
S3	1.41×10^{10}	2.58×10^{2}
S4	1.41×10^{10}	<30

Table A.16. Analysis of Rhesus Rotavirus Counts in ST1wST3 Monoreassortant Co-Infections

Co-Infection Type: ST1wST3 Monoreassortant (MRV-3DE Gene)	t Value	p Value	Degrees of Freedom
L1	-58.406	0.000	3
L2	-7.494	0.005	3
L3	-58.406	0.000	3
M1	-27.658	0.000	3
M2	-9.795	0.002	3
M3	-11.091	0.002	3
S1	-1.599	0.208	3
S2	-20.407	0.000	3
S3	-94.586	0.000	3
S4	-94.586	0.000	3

Table A.17. ST3wST2 Monoreassortant Infectious Particle Count from Rhesus Rotavirus Co-Infections

ST3wST2 Monoreassortant (MRV-2JO Gene)	ST3wST2 Single Infection Virus Count	ST3wST2 Co-Infection Virus Count
L1	3.83×10^{3}	9.15×10^{2}
L2	8.25×10^{3}	7.50×10^{3}
L3	3.73×10^{6}	3.91×10^{7}
M1	4.99×10^{3}	5.23×10^{5}
M2	6.06×10^{2}	<30
M3	3.75×10^{4}	6.12×10^{2}
S1	2.44×10^{5}	8.55×10^{3}
S2	4.09×10^{3}	<30
S3	7.29×10^{2}	1.40×10^{2}
S4	1.88×10^{4}	2.93×10^{4}

Table A.18. Analysis of ST3wST2 Monoreassortant Counts in Rhesus Rotavirus Co-Infections

ST3wST2 Monoreassortant (MRV-2JO Gene)	t value	p Value	Degrees of Freedom
L1	-8.610	0.003	3
L2	-0.469	0.671	3
L3	4.503	0.020	3
M1	11.620	0.001	3
M2	-7.091	0.006	3
M3	-114.863	0.000	3
S1	-13.976	0.001	3
S2	-9.798	0.002	3
S3	-9.207	0.003	3
S4	3.387	0.043	3

Table A.19. Rhesus Rotavirus Infectious Particle Count from ST3wST2 Monoreassortant Co-Infections

Co-Infection Type: ST3wST2 Monoreassortant (MRV-2JO Gene)	RRV Single Infection Virus Count	RRV Co-Infection Virus Count
L1	$2.57x10^7$	$5.04x10^5$
L2	$2.57x10^7$	$8.20x10^6$
L3	$3.41x10^5$	$4.81x10^4$
M1	$2.57x10^7$	$3.41x10^5$
M2	$2.57x10^7$	$3.45x10^5$
M3	$2.57x10^7$	$5.25x10^5$
S1	$2.57x10^7$	$4.03x10^4$
S2	$2.57x10^7$	<30
S3	$2.57x10^7$	<30
S4	$2.57x10^7$	$7.88x10^3$

Table A.20. Analysis of Rhesus Rotavirus Counts in ST3wST2 Monoreassortant Co-infections

Co-Infection Type: ST3wST2 Monoreassortant (MRV-2JO Gene)	t Value	p Value	Degrees of Freedom
L1	-25.977	0.000	3
L2	0.924	0.423	3
L3	-13.955	0.001	3
M1	-26.155	0.000	3
M2	-26.137	0.000	3
M3	-25.919	0.000	3
S1	-26.482	0.000	3
S2	-26.523	0.000	3
S3	-26.523	0.000	3
S4	-26.515	0.000	3

Table A.21. Reovirus MRV-1LA, MRV-2JO, and MRV-3DE Infectious Particle Count from Simian Rotavirus Co-Infections

Reovirus Type	Reovirus Single Infection Virus Count	Reovirus Co-Infection Virus Count
MRV-1LA	$9.24x10^4$	$8.86x10^4$
MRV-2JO	$4.20x10^7$	$7.29x10^6$
MRV-3DE	$5.98x10^4$	$4.31x10^4$

Table A.22. Analysis of Reovirus MRV-1LA, MRV-2JO, and MRV-3DE Counts from Simian Rotavirus Co-Infections

Reovirus Type	t Value	p Value	Degrees of Freedom
MRV-1LA	-0.897	0.436	3
MRV-2JO	-7.270	0.005	3
MRV-3DE	-5.714	0.011	3

Table A.23. Simian Rotavirus Infectious Particle Count from Reovirus MRV-1LA, MRV-2JO, and MRV-3DE Co-Infections

Co-Infection Type (Reovirus Serotype)	SA11 Single Infection Virus Count	SA11 Co-Infection Virus Count
MRV-1LA	$5.23x10^9$	$3.38x10^3$
MRV-2JO	$3.26x10^8$	$5.81x10^3$
MRV-3DE	$4.97x10^{10}$	$1.66x10^3$

Table A.24. Analysis of Simian Rotavirus Virus Counts in Reovirus MRV-1LA, MRV-2JO, and MRV-3DE Co-Infections

Co-Infection Type (Reovirus Serotype)	t Value	p Value	Degrees of Freedom
MRV-1LA	-5.596	0.001	3
MRV-2JO	-29.158	0.000	3
MRV-3DE	-15.507	0.001	3

Table A.25. Average Mouse Weights (in grams) from Single and Co-Infections with Rhesus Rotavirus and Reovirus

Treatment	Day 1	Day 7	Day 28	Day 56
MEM	4.12	8.26	18.28	24.28
RRV	4.49	8.03	16.69	22.18
MRV-1LA	3.51	6.65	17.03	24.74
MRV-1LA + RRV	3.42	7.50	18.08	26.01
RRV → MRV-1LA	4.21	8.07	18.27	24.72
MRV-3DE	4.16	8.34	17.58	24.31
MRV-3DE + RRV	4.05	7.76	15.65	22.05

Table A.26. Analysis of Mouse Weights from Days 7, 28, and 56 in Single and Co-Infections with Rhesus Rotavirus and Reovirus

Treatment	Day	t Value	p Value	Degrees of Freedom
MEM*RRV	7	0.188	0.860	4
MEM*RRV	28	0.955	0.394	4
MEM*RRV	56	1.085	0.339	4
MEM*MRV-1LA	7	1.917	0.128	4
MEM*MRV-1LA	28	0.632	0.561	4
MEM*MRV-1LA	56	-0.343	0.749	4
MEM*MRV-3DE	7	-0.058	0.956	4
MEM*MRV-3DE	28	0.396	0.712	4
MEM*MRV-3DE	56	-0.033	0.976	4
MEM*RRV+MRV-1LA	7	0.944	0.398	4
MEM*RRV+MRV-1LA	28	0.162	0.879	4
MEM*RRV+MRV-1LA	56	-1.414	0.230	4
MEM*RRV→MRV-1LA	7	0.203	0.849	4
MEM*RRV→MRV-1LA	28	0.010	0.992	4
MEM*RRV→MRV-1LA	56	-0.309	0.772	4

MEM*RRV+MRV-3DE	7	0.638	0.558	4
MEM*RRV+MRV-3DE	28	1.998	0.116	4
MEM*RRV+MRV-3DE	56	1.166	0.308	4

Table A.27. Percentage of Mice that Developed Diarrhea from Single and Co-Infections with Rhesus Rotavirus and Reovirus

Treatment	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
MEM	0	0	0	0	0	0	0
RRV	0	68	58	79	53	16	0
MRV-1LA	0	0	0	0	0	0	0
MRV-1LA + RRV	0	0	0	17	33	6	0
RRV → MRV-1LA	0	0	74	37	47	0	0
MRV-3DE	0	6	6	38	19	0	0
MRV-3DE + RRV	0	10	47	67	57	0	0

Table A.28. Analysis of Mouse Diarrhea Percentages from Days 1-7 in Single and Co-Infections with Rhesus Rotavirus and Reovirus

Treatment	Day	t Value	p Value	Degrees of Freedom
RRV*MRV-1LA	1	0.000	1.000	4
RRV*MRV-1LA	2	7.833	0.001	4
RRV*MRV-1LA	3	5.109	0.007	4
RRV*MRV-1LA	4	9.504	0.001	4
RRV*MRV-1LA	5	2.359	0.078	4
RRV*MRV-1LA	6	0.987	0.380	4
RRV*MRV-1LA	7	0.000	1.000	4
RRV*MRV-3DE	1	0.000	1.000	4
RRV*MRV-3DE	2	6.718	0.003	4
RRV*MRV-3DE	3	4.680	0.009	4
RRV*MRV-3DE	4	3.132	0.035	4
RRV*MRV-3DE	5	1.846	0.139	4
RRV*MRV-3DE	6	0.987	0.380	4
RRV*MRV-3DE	7	0.000	1.000	4
RRV*MRV-1LA+RRV	1	0.000	1.000	4
RRV*MRV-1LA+RRV	2	7.833	0.001	4
RRV*MRV-1LA+RRV	3	5.109	0.007	4

RRV*MRV-1LA+RRV	4	5.579	0.005	4
RRV*MRV-1LA+RRV	5	0.996	0.376	4
RRV*MRV-1LA+RRV	6	0.800	0.496	4
RRV*MRV-1LA+RRV	7	0.000	1.000	4
RRV*MRV-3DE+RRV	1	0.000	1.000	4
RRV*MRV-3DE+RRV	2	4.738	0.009	4
RRV*MRV-3DE+RRV	3	0.815	0.461	4
RRV*MRV-3DE+RRV	4	0.764	0.487	4
RRV*MRV-3DE+RRV	5	0.424	0.693	4
RRV*MRV-3DE+RRV	6	0.987	0.380	4
RRV*MRV-3DE+RRV	7	0.000	1.000	4
RRV*RRV→MRV-1LA	1	0.000	1.000	4
RRV*RRV→MRV-1LA	2	7.833	0.001	4
RRV*RRV→MRV-1LA	3	-0.771	0.484	4
RRV*RRV→MRV-1LA	4	1.988	0.118	4
RRV*RRV→MRV-1LA	5	0.423	0.694	4
RRV*RRV→MRV-1LA	6	0.987	0.380	4
RRV*RRV→MRV-1LA	7	0.000	1.000	4
MEM*RRV	1	0.000	1.000	4
MEM*RRV	2	-7.833	0.001	4
MEM*RRV	3	-5.109	0.007	4
MEM*RRV	4	-9.504	0.001	4
MEM*RRV	5	-2.359	0.078	4
MEM*RRV	6	-0.987	0.380	4
MEM*RRV	7	0.000	1.000	4
MRV-1LA+RRV*MRV-3DE+RRV	1	0.000	1.000	4
MRV-1LA+RRV*MRV-3DE+RRV	2	-1.686	0.167	4

MRV-1LA+RRV*MRV-3DE+RRV	3	-3.553	0.024	4
MRV-1LA+RRV*MRV-3DE+RRV	4	-2.137	0.099	4
MRV-1LA+RRV*MRV-3DE+RRV	5	-0.828	0.454	4
MRV-1LA+RRV*MRV-3DE+RRV	6	1.032	0.360	4
MRV-1LA+RRV*MRV-3DE+RRV	7	0.000	1.000	4
RRV→MRV-1LA*MRV-1LA+RRV	1	0.000	1.000	4
RRV→MRV-1LA*MRV-1LA+RRV	2	0.000	1.000	4
RRV→MRV-1LA*MRV-1LA+RRV	3	6.998	0.002	4
RRV→MRV-1LA*MRV-1LA+RRV	4	1.432	0.225	4
RRV→MRV-1LA*MRV-1LA+RRV	5	0.603	0.579	4
RRV→MRV-1LA*MRV-1LA+RRV	6	-1.032	0.360	4
RRV→MRV-1LA*MRV-1LA+RRV	7	0.000	1.000	4

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BIOGRAPHICAL INFORMATION

Melody completed her undergraduate work and received her Bachelors of Science degree in biology from The University of Texas at Arlington in 2003. A year after receiving her Bachelors degree she soon began her graduate studies in virology at UTA. During her graduate career, she got married, had a daughter, and joined her husband in his long tortuous journey fighting refractory Hodgkin's Lymphoma, all while never missing a deadline. In August 2010, she received her PhD in Quantitative Biology, under Dr. Michael Roner's tutelage. She would like to work in academia and research facilities focusing on oncology and virology research. She wants to use her knowledge and training to study viruses and find new successful viral based cancer treatments, specifically for cancers that don't respond to traditional chemotherapy drugs.