ANTIVIRAL ACTIVITY OF PRIMARY HUMAN TROPHOBLAST CONDITIONED MEDIA AGAINST RIFT VALLEY FEVER VIRUS

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ABSTRACT

Rift Valley Fever Virus (RVFV) is a Phlebovirus that is found across most of Africa with the majority of cases coming from eastern Africa near Egypt to as far south as South Africa and is a significant public health concern. *Aedes* mosquitos are the primary vectors of the disease, and as a consequence of this cases are more common after heavy rainfall or flooding. RVFV primarily infects domesticated ruminants, and epidemics are characterized by abortion storms among livestock. During these epidemics, people inevitably come into contact with infected tissues and these people are at the highest risk for zoonosis. Domestic ruminants are not the only animals at risk for spontaneous abortion or juvenile death; wild ruminants and other animals also develop similar outcomes. RVFV is known to cause extensive fetal loss in animals but a similar outcome in cases of human infections is not well documented. Two published case reports exist which suggest vertical transmission events could occur in humans. Currently there are only speculative *in vitro* models investigating the potential for the vertical transmission of RVFV have been demonstrated. Primary Human Trophoblasts (PHT) cells isolated from human placentas could serve as a good model for a vertical transmission event because these cells serve as a physical barrier to the placenta and have also been shown to be resistant to viral infection. PHT cells express a very unique cluster of miRNA's located on chromosome 19 and the

expression of these miRNA's is specific to pregnancy with expression dropping off shortly after birth. The viral resistance seen in PHT cells can also be conferred to other cell types. One possible way that viral resistance can be conferred to other cell types is via exosome. Since RVFV is of public health concern and potentially associated with fetal infections in humans, we will test the ability of primary human trophoblast conditioned media (PHTCM) to confer viral resistance to other cell types against RVFV.

TABLE OF CONTENTS

LIST OF FIGURES

1.0 INTRODUCTION

Rift valley fever virus (RVFV) is an enveloped, single stranded negative sense RNA virus which belongs to the Bunyaviridae family and the Phlebovirus genus[1]. The RVFV genome is composed of three segments that are designated L, M and S of negative or ambisense polarity. The L segment of the genome encodes the viral RNA-dependent RNA polymerase. The M segment encodes for the surface glycoproteins annotated G_n and G_c as well as NSm 1 and 2. Finally the S segment is ambisense allowing it to code for the nucleoprotein N and the non-structural protein NSs [9]. The complete viral particle measures 90-110 nm in diameter and has a lipid membrane that presents two glycoproteins [9].

The geographic distribution of RVFV encompasses most of Africa from Egypt to South Africa with the majority of reports coming from eastern and southern Africa. *Aedes* mosquitos are the primary vectors, and cases of RVFV are particularly prominent during times of unusually heavy rainfall and flooding. RVFV infects livestock as well as humans with the most dramatic impact being observed on livestock in the form of spontaneous abortion and juvenile death. These “abortion storms” affect nearly 100% of pregnant animals during an epidemic[1].

Currently, the clinical disease phenotype that leads to vertical transmission and abortion in livestock has not been well characterized in humans [8]. There are two cases documented, one in 2006 and the other in 2008, which were suspected to be vertical transmission events of RVFV from mother to child [3,4].

1.1 RIFT VALLEY FEVER VIRUS

1.1.1 Epidemiology

Rift Valley Fever (RVF) is a mosquito-borne viral zoonosis that was first isolated and characterized in 1931 in Kenya [14]. RVFV zoonosis occurred primarily in Kenya until the disease was recognized in South Africa in 1951, when humans became ill after handling dead and infected animals. More cases were later confirmed in Zimbabwe, Zambia, the Sudan, and other east African countries. In 1977, there was a major epidemic in Egypt, with 20–40,000 human clinical illnesses and 600 human deaths. RVF was then identified in West Africa in Senegal and Mauritania, where human mortality was again high. In 2000, an outbreak occurred in Saudi Arabia marking the first occurrence RVFV outside Africa [13]. During 2007 a large RVF outbreak occurred in Sudan with a total of 747 confirmed human cases including 230 deaths, although it is thought that over 75,000 were infected during this time. The outbreak was most severe near the White Nile and the Blue Nile Rivers [16].

1.1.2 Transmission

Mosquitoes in the *Aedes* genus have been considered as the reservoir, as well as vectors, since their transovarially infected eggs withstand desiccation and larvae hatch when in contact with water. However, different mosquito species can serve as vectors for RVF, creating a complex epidemiologic pattern in East Africa[10]. Currently RVFV has been isolated from over 30

species of mosquitoes in six genera [15]. Infection by RVF usually spreads among livestock first through mosquito bites. In addition, the infection can also be transmitted vertically between domesticated animals[11]. Examples of human exposures associated with acute RVF infection include animal contact, consuming or handling products from sick animals, consuming raw milk, milking, skinning, slaughtering, sleeping with animal herds, touching blood, and caring for animals during birthing, and socio-demographic factors (male gender and herdsperson occupation), Non-animal related risks include living in proximity to water sources and having a flooded home[17]. RVFV has also been shown to be highly infectious via aerosol although the extent of the role it plays in epidemics is unknown[1].

1.1.3 Pathogenesis

Human infection with RVFV is generally asymptomatic, and the majority of those with clinical symptoms present with a short febrile illness[21]. The incubation period for RVFV is 2 to 6 days and symptoms start abruptly with severe chills, malaise, dizziness, weakness, severe headache and nausea but usually resolve without sequelae[2]. Some of those cases result in recurring fevers with headaches for up to 10 days followed by two weeks of weakness before recovery[21]. A subset of infected patients will develop severe forms of disease including neurological disorders, vision loss, hemorrhagic fever and thrombosis. The overall fatality rate is estimated at 0.5-1% but in patients whose severe clinical outcomes the fatality rate has been reported to be as high as 29%[20].

1.2 RIFT VALLEY FEVER AND HUMAN PREGNANCY

1.2.1 Background

It is important to highlight the fact that aborted fetal materials and placental membranes could contain large numbers of virus particles capable of contaminating the local environment directly or infect animals in close contact[9]. Since animals accumulate large numbers of viral particles in their placentas then the question is whether or not we see something similar in humans. Human placentas potentially have a unique mechanism for protecting the fetus from viral infection.

During the 2000–2001 RVFV outbreak in the Arabian Peninsula, no child under the age of 10 years old was confirmed to have died as a result of RVFV infection. The underlying difference in susceptibility of young and pregnant animals with that observed in humans requires further study and raises an important question: are the dramatic differences in lethality the result of a lack of contact of children with infected mosquitoes or infected animals or are there true differences in susceptibility between young animals and young children [9]?

1.2.2 Case Reports

In 2006, a paper was published documenting a 5-day-old child who was admitted to intensive care with sepsis in September of 2000. The parents reported fever, difficulty breathing, and diminished activity on day 2 of life. The child was born at home in an area of Saudi Arabia affected by the ongoing RVFV outbreak. The mother was ill 4 days before delivery with symptoms including fever, headache, muscle aches, and dizziness. Six out of the nine family members of the mothers' family also became ill in the two weeks prior to her delivery, and the

grandfather even died of a confirmed RVF case. The family also owned sheep and goats that everyone had contact with during the epidemic resulting in their animals becoming ill and aborting. The child succumbed to disease with symptoms such as an enlarging liver, coagulopathy, anemia, poor liver function and RVFV-IgG positive. Since RVFV-IgM, which is not passed across the placenta, was positive in the child on the second day of life, it is strongly suspected that this was a case of vertical transmission from the mother[3].

In 2008, a second case report documents a pregnant Sudanese woman. The woman delivered a baby with an Apgar score of 5/10; the baby had a palpable liver and spleen as well as a skin rash. Samples were taken from the mother and the neonate both of whom tested positive for RVF-IgM. On the third day of life the neonate developed clinical jaundice and unfortunately it was at this point that the husband went against medical advice and discharged the mother and child from the hospital. This paper also includes an interesting comment stating that severe forms of RVFV with high maternal mortality have been observed in central Sudan but that the work remains unpublished[4].

1.2.3 Primary Human Trophoblast Cells

Primary human Trophoblasts (PHT) derived from human placentas may be useful in generating a model that represents trans-placental RVFV infection in humans. While PHT cells are known to form the physical barrier of the placenta, it is not well understood how they influence viral infections. PHT cells have not only been demonstrated to be resistant to viral infection, but their resistance can be conferred to nontrophoblast cells when incubated with conditioned medium from PHT cells[5, 6]. This affect has been shown with viruses including HIV, VZV, EEEV, VEEV, CHIKV, SINV, Rubella, CVB, PV, VSV, VV, HSV-1 and CMV[5, 6].

Certain products of PHT cells, for example C19MC miRNA's, are unique to pregnancy and are only expressed at high levels during the course of pregnancy. C19MC miRNA levels in the blood decline 24hrs after birth and are therefore likely a mechanism of protection for the unborn fetus. The C19MC is the largest cluster of miRNAs in the human genome[7] and the components of PHTCM that carry them are the exosomes. PHT-derived C19MC miRNA–containing exosomes may target a subpopulation of maternal cells and cause antiviral responses such as autophagy[6].

Since RVF is known to cause extensive fetal loss in animals, and it has the potential for fetal infection in humans, it is important to investigate the interaction between placental trophoblast cells and the virus.

2.0 STATEMENT OF THE PROJECT AND SPECIFIC AIMS

The aim of project will be to create a representative model for the transmissibility of RVFV across the placenta during pregnancy. By using media that has been conditioned on primary human trophoblast cells, we can test our hypothesis that *Rift Valley Fever Virus is not susceptible to the innate antiviral components of placental trophoblast cells.* By this reasoning, we predict that resistance of RVFV to the antiviral effects of trophoblast cells may be an explanation for the fetal loss in pregnant animals and possibly humans. By infecting cells in the presence of PHTCM and using the appropriate controls we can quantify the amount of antiviral activity conferred by PHTCM.

Aim 1: To quantify the anti viral affect of PHTCM against RVFV.

We will determine a constant number of cells to be infected as well as the appropriate MOI's to be used. Viral titers will be calculated after RVFV infection of cells in the presence or absence of PHTCM by preforming plaque assays and RT-PCR on the supernatants of infected cells. Based on our results, we will identify differences in the anti-viral activity of PHTCM between donors as well as the cell line that serves as the best model for infection.

Aim 2: To determine whether exosomes within PHTCM may contribute to the observed antiviral activity. By sonicating the PHTCM in order to destroy the exosomes within it we can determine if the exosomes play a role in conferring viral resistance to the recipient cells.

3.0 MATERIALS AND METHODS

3.1 BIOSAFETY

All experiments using live ZH501 RVFV were performed in the biosafety level 3+ Regional Biocontainment Laboratory (RBL) at the University of Pittsburgh. Powered air purifying respirators (PAPRs) were worn for respiratory protection and all work was conducted in a class II biosafety cabinet using Vesphene IIse (diluted 1:128, Steris Corporation, cat. #646101) as a disinfectant. Work involving MP-12 RVFV was performed under BSL-2 conditions in a class II biosafety cabinet, using Vesphene IIse (1:128).

3.2 CELL CULTURE

Vero E6 and A549 cells were cultured in an incubator set at 37°C and 5% CO_2 in Dulbecco's Modified Eagle Medium with 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% L-Glutamine. Cells were split with 0.05% trypsin/EDTA every 3 to 4 days upon reaching nearly 100% confluence. 1/12 of the total cells were then seeded into a new T-75 cell culture flask.

3.3 EXPERIMENTAL DESIGN

We chose a 96 well tissue culture plate so that each infection group could be done in triplicate. Each well at 100% confluence would contain about 100,000 cells and a hemocytometer was used to ensure that each well was seeded with a uniform quantity of cells. Based on the size of the wells and the 22-24 hours doubling times of the cells, it was determined that the wells should be seeded with 20,000 cells each.

Twenty-four hours after seeding cells the original D10 media was removed and replaced with 50ul of PHTCM from Dr. Coynes lab or 50ul of D2 media as control. D2 is Dulbecco's Modified Eagle Medium with 2% fetal bovine serum, 1% penicillin/streptomycin, and 1% L-Glutamine.

The cells were allowed to interact with the PHTCM for twenty-four hours before they were infected with RVFV. The infection groups we chose were MOI's of 0.1 and 0.01 (10,000 and 1,000 plaque forming units, respectively) per infection. The MOI's were diluted from stock viruses and delivered in 50µl volumes leaving a total of 100µl per well. The cells were then incubated at 37°C and 5% CO_2 for forty-eight hours after which the supernatant was harvested, diluted to 10^{-1} in 900ul of D2 media, then frozen at -80°C for future analysis by rt-PCR and plaque assay.

3.4 RNA EXTRACTION

The frozen supernatants samples from infections were thawed and 100ul was removed from each to be inactivated and used for RNA extraction. The 100ul of the samples were added

to 900ul of TriReagent. The samples were mixed and allowed to sit for 5 minutes at which point the virus was inactivated.

After inactivation 200ul of chloroform was added to each sample and the tubes were mixed by inversion for twenty seconds and then left to sit for 3 minutes. The samples were then centrifuged at 12,000 x G for fifteen minutes at 4°C. After centrifugation the clear aqueous phase separated and rose to the top. The aqueous phase was then carefully pipeted off and transferred to a new tube. 500ul of 70% EtOH was then added to the aqueous phase and vortexed. The resulting lysates were then transferred to a viral spin column from a PureLink® Viral RNA/DNA Mini Kit. The columns were then washed with the provided wash buffer with ethanol twice and then centrifuged again to dry the column. The columns were finally placed in RNase free recovery tubes and 40ul of RNase-free water was added to the column. The water was allowed to sit for a minute at room temperature and then centrifuged to elute the RNA. The purified RNA was stored at -80°C for future analysis by rt-PCR.

3.5 RT-PCR

PCR was setup in a 96 well plate and each well was prepared with 20ul of master mix containing 62.5% Invitrogen 2x reaction mix, 2.5% RVFV-2912Fwd primer, 2.5% RVFV-2981Rev primer, 2.5% RVFV-2950-probe, 2.5% Invitrogen Superscript™III RT/Platinum® Taq mix and 27.5% Invitrogen DEPC water. After the master mix was added 5ul of DEPC water was added to the no-template control wells and 5ul of each of the standards and unknowns were added to the wells in duplicate. The wells were finally sealed with a clear plastic film and placed in the rt-PCR machine and run for 40 cycles.

3.6 PLAQUE ASSAY

Vero E6 cells were seeded into 6 well plates with D10 media and were incubated for 24 hours until reaching >95% confluence. Once the Vero E6 cells reached confluence supernatant samples from the initial infection experiments were thawed and serially diluted in D2 media for infection. The media on the Vero E6 cells was removed and 200ul of the appropriate infection dilution was added. The plates were placed in the 37°C incubator and rocked every 10 minutes for an hour. After the 1-hour infection period the infection media was removed and replaced with a Nutrient agar. The nutrient agar was comprised of Dulbecco's 1X minimum essential medium, 2% FBS, 1.5% HEPES buffer, 1% penicillin/streptomycin and 0.73% SeaKem agrose. After the nutrient agar had solidified the plates were allowed to incubate at 37°C and 5% CO_2 for 72 hours. Once the plaques have formed 37% formaldehyde was added to each and left to sit for at least 3 hours to completely inactivate the virus. The formaldehyde and agar was then removed and the plates were rinsed. Finally 0.1% crystal violet was added to the wells so that plaques could be counted and viral titers could be calculated.

3.7 SONICATION

Sonication was preformed with a Branson 450 Sonifier and samples of PHTCM were sonicated in a high intensity cup horn. The output was 20kHz set at 80% amplitude and our sample of PHTCM was sonicated 3 times for 15 seconds each.

3.8 STATISTICAL ANALYSIS

All statistical significance was calculated in Graph Pad Prism version 6.0. Significance was determined by a one-way analysis of variance with a Sidak multiple comparisons analysis at a 95% confidence interval.

4.0 RESULTS

4.1 AIM 1: TO QUANTIFY THE ANTI VIRAL AFFECT OF PHTCM AGAINST RVFV

There is currently no established *in vitro* model for trans-placental infection with RVFV and it was necessary to develop a protocol for our analysis. The first goal was to choose a cell type to create a model for infection. Vero E6 cells are known to be very susceptible to RVFV infection and are often used to propagate RVFV stock. The conditioned media is cultured on PHT cells that are human in origin and it was uncertain whether or not the PHTCM would be able to confer resistance to the Vero E6 cells. The pilot experiment was carried out on Vero E6 cells and the PHTCM used was from the first sample received from Dr. Carolyn Coyne's laboratory. MP12 virus, an attenuated vaccine strain, was the first RVFV strain used at MOI's of 0.1 and 0.01 during the initial infection.

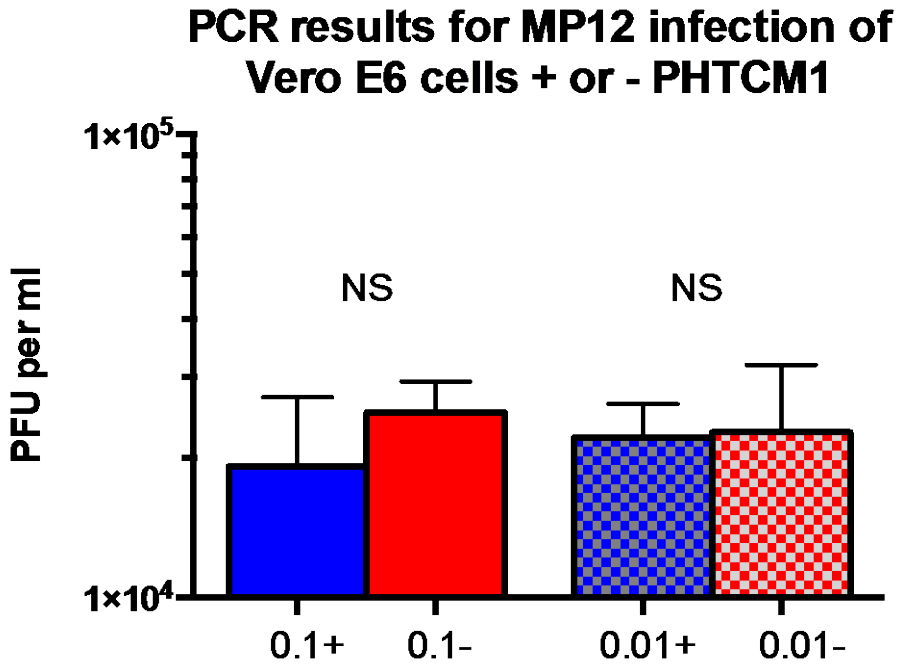

Figure 1. Determining the antiviral affect of PHTCM1 on the MP12 virus infection of Vero E6 cells

(P-values were 0.71 for the 0.1 MOI group and 0.97 for the 0.01 MOI group)

The results shown in Figure 1 represent the viral titers of the supernatants 48 hours after the initial infection with MP12 virus. The titers were calculated by plaque assay and each column represent triplicate data with blue columns representing infections in the presence of PHTCM1 and red columns representing the controls. Based on this initial experiment there was no significant difference in the infection of Vero E6 cells with or without PHTCM1. There are also no observable trends suggesting that PHTCM1 did not have an effect on viral titer.

Moving forward, we decided to switch from RVFV MP12 to Zh501. The Zh501 strain of RVFV was originally isolated from a human patient during the outbreak of 1977 in Egypt[18].

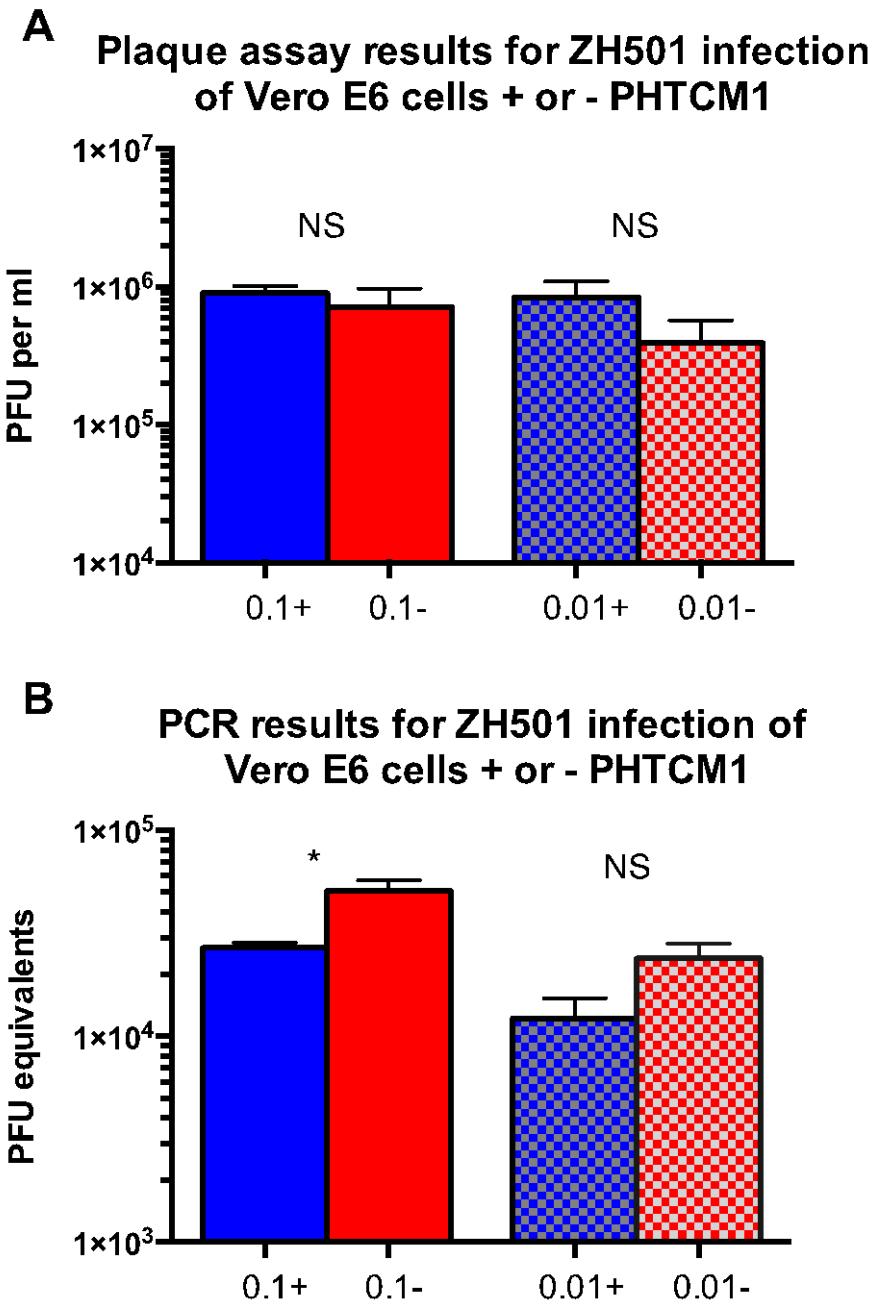

Figure 2. Determining the antiviral affect of PHTCM1 on the ZH501 virus infection of Vero E6 cells.

Figure (A) represents viral titer calculated by plaque assay, the P values were 0.53 for the 0.1 MOI group and 0.06 for the 0.01 MOI group. Figure (B) represents viral titer calculated by RT-PCR the P values were 0.04 for the 0.1 MOI group and 0.19 for the 0.01 MOI group.

Vero E6 cells were infected with RVFV ZH501 with or without PHTCM1 and the results shown above in Figure 2a and 2b represent plaque assay and PCR data for the experiment. Figure 2a, which represents the plaque assay data, shows no statistically significant differences in viral titers on Vero E6 cells with or without PHTCM. Figure 2b, which represents the PCR data, shows minimal statistical significance with a P-value of 0.043 in the 0.1 MOI infection group and a trend towards reduction in viral titer in the 0.01 MOI group. Based on the data it seems as though PHTCM1 is either not able to efficiently confer viral resistance to Vero E6 cells or it is not effective against RVFV MP12 and ZH501.

Our next step in the investigation was to try a new cell type based on the data we had collected on Vero E6 cells. We decided this time to use A549 cells which is a human cell line and as such may be more receptive to the antiviral components of PHTCM. The conditioned media we initially received from Dr. Coyne was not enough for another experiment in triplicate so we requested a new sample. It should be noted that this sample, which will be referred to as PHTCM2, was from a different donor's placental cells.

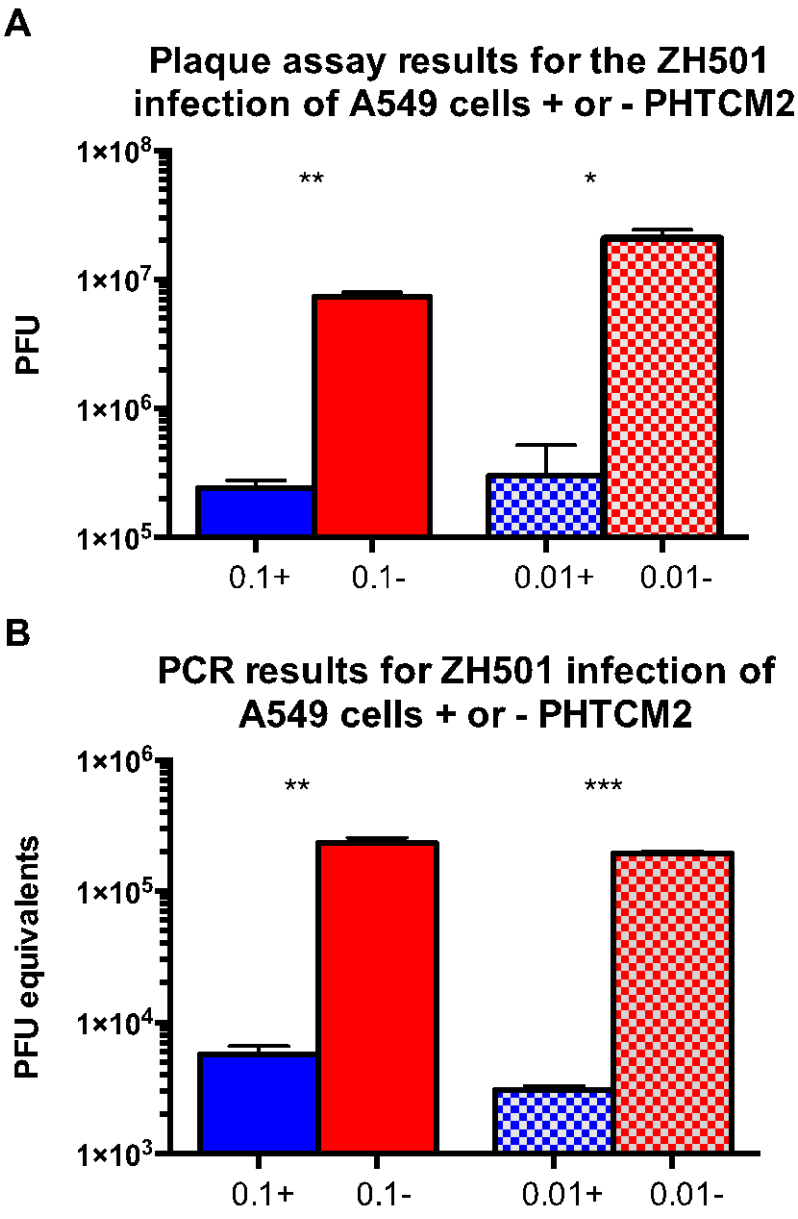

Figure 3. Determining the antiviral affect of PHTCM2 on the ZH501 virus infection of A549 cells.

Figure (A) represents viral titer calculated by plaque assay, the P values were 0.005 for the 0.1 MOI group and 0.009 for the 0.01 MOI group. Figure (B) represents viral titer calculated by RT-PCR the P values were 0.006 for the 0.1 MOI group and 0.0005 for the 0.01 MOI group.

A549 cells were infected with RVFV ZH501 with or without PHTCM1 and the results shown above in Figure 3a and 3b represent plaque assay and PCR data for the experiment. The data in figures 3a and 3b both show high levels of statistical significance and nearly a two log reductions in viral titer. A two log reduction equates to a 99% reduction in viral titer meaning that PHTCM2 was highly effective at inhibiting RVFV on A549 cells. What was not known at this point was whether changing the cell type or changing the PHTCM resulted in significant viral reduction.

Next we wanted to investigate which variable, cell type or PHTCM, impacted the acute change in results between experiments 2 and 3. The fact that we did not have any PHTCM1 remaining to test on A549 cells as a direct comparison of conditioned medias meant that we could only change the cell type. By repeating the experiment with Vero E6 cells and PHTCM2 we can determine which variable is more important to the extent of antiviral activity observed.

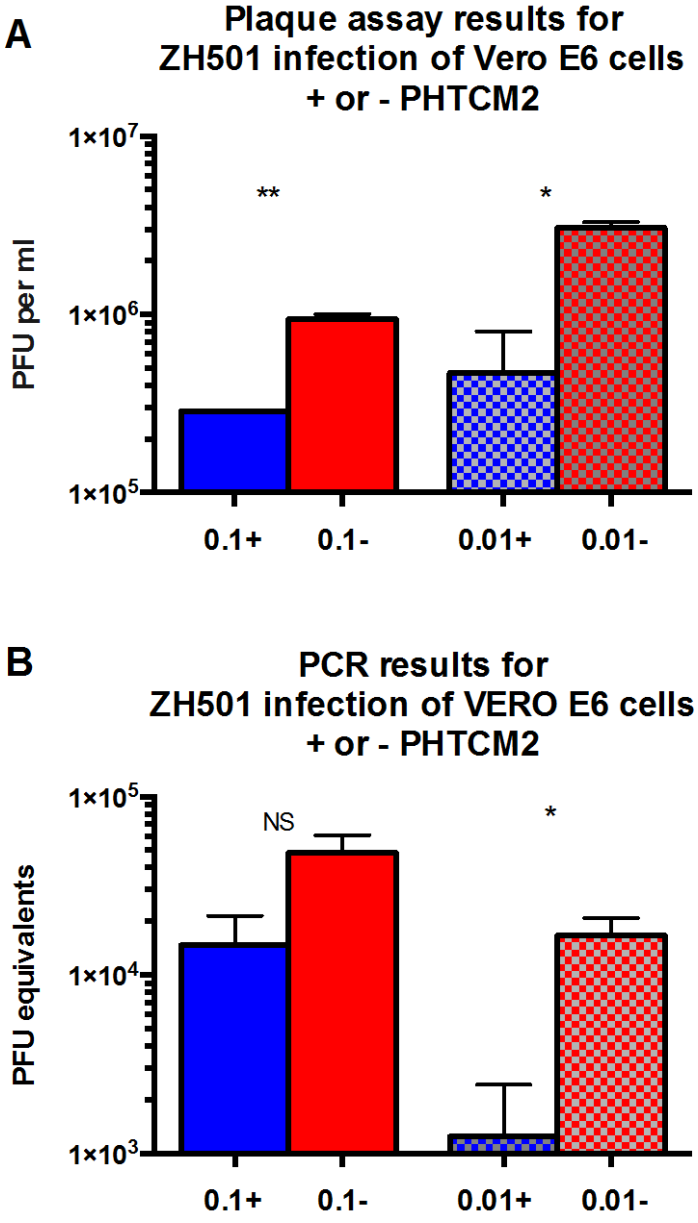

Figure 4. Determining the antiviral affect of PHTCM2 on the ZH501 virus infection of Vero E6 cells.

Figure (A) represents viral titer calculated by plaque assay, the P values were 0.005 for the 0.1 MOI group and 0.024 for the 0.01 MOI group. Figure (B) represents viral titer calculated by RT-PCR the P values were 0.08 for the 0.1 MOI group and 0.04 for the 0.01 MOI group.

Figures 4a and 4b show significant reductions in viral titer in the presence of PHTCM2 with the exception of the PCR data for the 0.1 MOI group. This data contrasts the results we observed in Figures 2a and 2b that looked at Vero E6 cells and PHTCM1. This contrasting result would seem to suggest that the PHTCM1 is not as effective as PHTCM2 at conferring viral resistance. The comparison between figures 2 and 4 also tells us that PHTCM is able to confer resistance to Vero E6 cells.

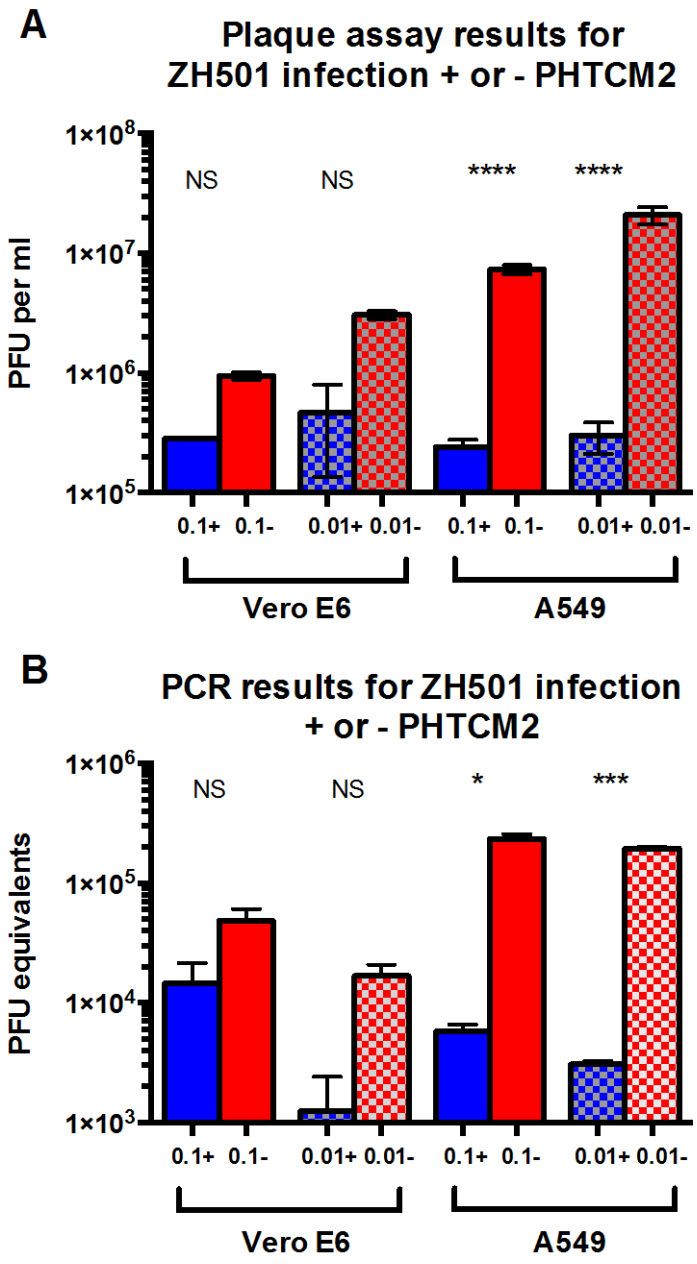

Figure 5. Comparing the effectiveness of PHTCM by cell type.

Figure (A) represents viral titer calculated by plaque assay, the P values from left to right were 0.95, 0.08, <0.0001 and <0.0001 Figure (B) represents viral titer calculated by RT-PCR the P values from left to right were 0.16, 0.08, 0.012 and 0.001.

Figure 5 is a direct comparison of Vero E6 and A549 cells by pooling the data and visually demonstrates the fact that PHTCM most effectively confers resistance to A549 cells. A549 cells also appear to be innately more susceptible to infection based on the higher levels of virus found in the control groups when compared to Vero E6 controls. Based on the data A549 cells will be used as our recipient cell type moving forward.

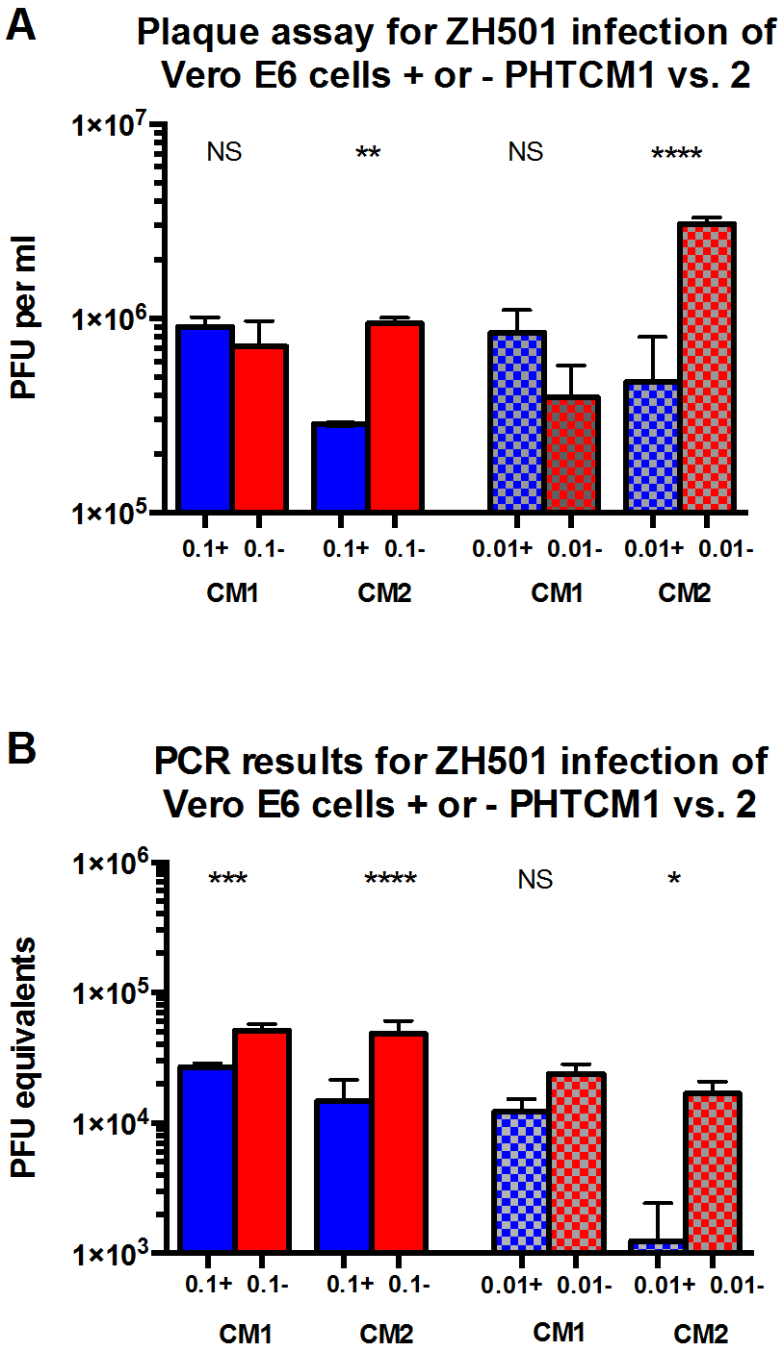

Figure 6. Comparing the effectiveness of PHTCM1 vs. 2 on Vero E6 cells.

Figure (A) represents viral titer calculated by plaque assay, the P values from left to right were 0.75, 0.006, 0.067 and <0.0001 Figure (B) represents viral titer calculated by RT-PCR the P values from left to right were 0.0007, <0.0001, 0.12 and 0.03.

Figure 6 shows a direct comparison of the data from all Vero E6 infections because they had been tested with both PHTCM1 and 2. The aim of this figure was to identify which PHTCM was best able to confer viral resistance. Based on the data PHTCM2 is better able to confer viral resistance based on visual trends and, in the case of the 0.01 MOI groups, statistical significance.

4.2 AIM 2: TO DETERMINE WHETHER EXOSOMES WITHIN PHTCM MAY CONTRIBUTE TO THE OBSERVED ANTIVIRAL ACTIVITY.

Based on Figures 5 and 6 we can conclude that A549 cells and PHTCM2 will serve as the best model for RVFV infection. With a reliable model we next wanted to try to identify a component of PHTCM that was contributing to the antiviral activity we had observed. A paper published on PHTCM and viral resistance suggested that exosomes present in the PHTCM functioned as a mediator in viral resistance. Treating the PHTCM with sonication was shown to completely eliminate the antiviral effect of PHTCM[6]. Sonication uses of ultrasonic frequencies too agitate particles within a solution and in this case would have destroyed any exosomes within the PHTCM. We wanted to test the exosome mediated antiviral activity in the case of RVFV infection so we sonicated a sample of PHTCM2 and set up an experiment with 3 groups: PHTCM, PHTCM that had been sonicated and a control with unconditioned D2 media.

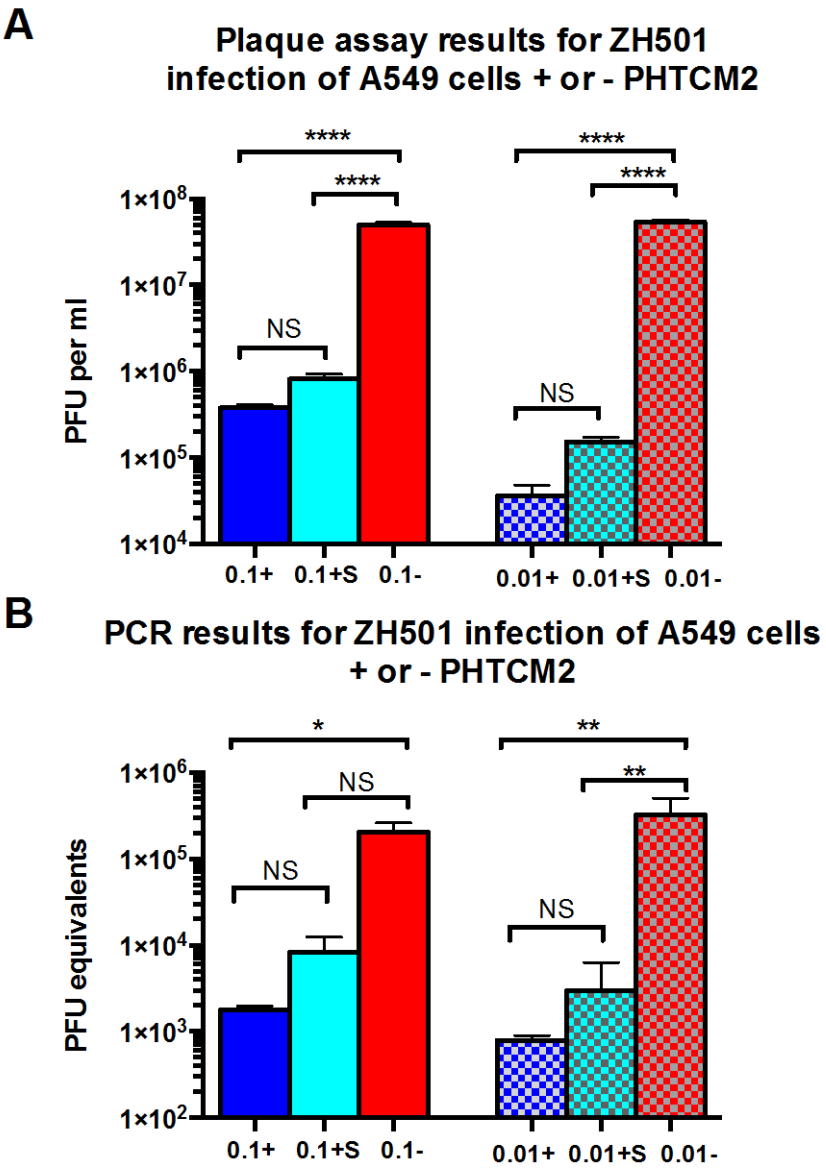

Figure 7. Determining the role of exosomes in the conferred resistance by PHTCM2.

0.1 and 0.01 represent MOIs and + or – represents the presence of PHTCM. The +S represents sonicated PHTCM. Figure (A) represents viral titer calculated by plaque assay, the P values from left to right were 0.99 <0.0001, <0.0001, 0.99 <0.0001 and <0.0001. Figure (B) represents viral titer calculated by RT-PCR the P values from left to right were 0.99 <0.055, <0.046, 0.99 <0.0019, <0.0017.

Figures 7a and 7b represent the data from an experiment in which a sample of PHTCM was sonicated and compared with untreated PHTCM and an unconditioned D2 media control. The results show a visual trend that does suggest sonicating PHTCM inhibits its ability to confer viral resistance. The exosomes are therefore likely to play a role in the inhibition of RVFV infection.

5.0 DISCUSSION

Rift Valley Fever Virus remains an important disease of both humans and animals in eastern Africa and parts of the Middle East. It is important to study this viral zoonosis because the range of the virus is expanding with subtle changes in climate and the ability to be spread by new mosquito vectors. The economic loss and disease burden would be extremely high should the virus travel further north through the middle east and into Europe. The possibility of the virus making its way across the Atlantic Ocean to the United States would also prove to be a devastating problem. The potential for this virus to spread to other parts of the world is real and therefore it is necessary to take preventative action now by researching vaccines, treatments and disease pathology.

The most dramatic disease outcomes that we see in animals, specifically domesticated ruminants, are the abortion storms and juvenile death. Spontaneous abortion can be seen in nearly 100% of pregnant animals during an outbreak and the disease is also highly pathogenic in juveniles. These kind of epidemic events lead to huge economic losses for farming communities and inevitably result in humans coming in contact with the disease. The close interaction of the farming communities and their animals means that many of the individuals near an outbreak of RVFV will come in to contact with some form of infected materials. The dramatic impact we see on young and pregnant animals raises the question of why we don’t see similar disease phenotypes in humans.

RVFV has been implicated in two published case studies demonstrating vertical transmission events but outside of these cases little information exists. There have been serological studies on the prevalence of RVFV antibodies in the serum of women, for example one study in Mozambique. The researchers took advantage of a nation-wide study of maternal morbidity from 1981-83 that collected serum samples. ELISAs were used to look for RVFV IgG antibodies in the women's sera and it was found that mothers experiencing fetal death or miscarriage had the same RVFV antibody prevalence as those with normal deliveries. The only published data on RVFV outbreaks in Mozambique at the time was a small outbreak among cattle in 1969 [8]. Although there was no correlation found between sera RVFV antibodies and fetal death the cohort in the study may not be the most applicable because of a limited history of endemic RVFV.

Conditioned medium from Primary Human Trophoblast cells is broadly antiviral, inhibiting infection by viruses known to cause perinatal infection in human beings. The viral resistance can also be conferred to nontrophoblast cells by exposing them to PHTCM[19]. For these reasons we felt that using PHTCM would create a good model for vertical transmission events associated with RVFV infection.

Vero E6 cells are commonly used to passage RVFV as the virus easily infects them and we thought that they would be a good place to start. Our initial experiment was designed to investigate the ability of PHTCM to confer resistance to Vero E6 cells when infected with RVFV MP12 but the results were inconclusive. MOI's of both 0.1 and 0.01 resulted in no significant differences in viral titer by plaque assay coinciding with our initial hypothesis that The question that arose was whether the PHTCM was failing to confer resistance to the Vero E6 cells or if RVFV was not susceptible to the type of resistance being conferred by PHTCM1.

The next step in investigating our hypothesis was to change our virus from MP12 to the fully virulent ZH501 strain. There was a possibility that the PHTCM1 could act on virulence factors of RVFV ZH501 that are not present in RVFV MP12. The results of this experiment were similar in terms of viral inhibition and ZH501 was shown to be clearly more virulent as titers increased by a log and a half. Interestingly our PCR data showed a visual trend toward viral inhibition and the difference in the 0.1 MOI group was minimally statistically significant. Because RVFV did not seem to be affected by the PHTCM1 on Vero E6 cells regardless of virus strain we wanted to see if a different cell line would show similar results. We wanted to change the cell type because there was still the possibility that the viral resistance from PHTCM was not being effectively conferred to Vero E6 cells.

A549 cells are human lung adenocarcinoma cells and we thought they might respond to the human PHTCM more effectively than the African green monkey Vero E6 cells. We set up our experiment with the A549 cells this time but we had to request a new sample of PHTCM as well since we had depleted the original. The parameters for the experiment were the same with the exception of cell type and PHTCM batch but the results were dramatically different. This time we were able to show a nearly 2 log, or 99%, reduction in viral titer in the presence of PHTCM. All of the data by plaque assay and PCR was very significant and demonstrated very clearly that PHTCM2 can effectively confer viral resistance to A549 cells. This was the first evidence that went against our hypothesis. Because we changed two variables, PHTCM batch and cell type in the last experiment we wanted to identify which variable played the biggest role in the change of result. Unfortunately we couldn't go back to PHTCM1 as our supply had been exhausted so we went back to Vero E6 cells this time with PHTCM2.

The results of our experiment with Vero E6 cells and PHTCM2 were much different from the first experiment with PHTCM1. This time viral resistance was conferred to Vero E6 cells albeit to a lesser extent than with A549 cells. About a half a log reduction was observed across plaque assay and rt-PCR results. The results were, for the most part, significant with the exception of the PCR data for the 0.1 MOI group. This failure to achieve significance can be attributed to a slight outlier in the data set however. The results of this experiment suggested two things; first that PHTCM2 outperforms PHTCM1 and second that PHTCM overall is better able to confer viral resistance to A549 cells than Vero E6 cells. Figures 5 and 6 are shown in the results specifically to highlight this point through direct comparisons of all the data.

We felt confident that we had established a model for infection that clearly disproved our hypothesis. The fact that PHTCM is able to reduce viral titer by 99% *in vitro* could be demonstrating the reason why we have not seen many vertical transmission events in humans up until this point. The fact remains that vertical transmission and abortion is a very prominent disease phenotype in some animal species, and therefore must be unique characteristics of the human placenta which protects the fetus.

The literature on PHTCM has demonstrated that a specific cluster of miRNAs on Chromosome 19, which is unique to humans, could be responsible for the protective antiviral activity *in vivo*. Exosomes containing these miRNAs can be found circulating in maternal and fetal blood supplies throughout pregnancy. The levels of these endosomes quickly drops off after birth, which is another finding supporting the protective role of the endosomes. This mechanism *in vivo* could be similar to the mechanism we see *in vitro* which allows viral resistance to be conferred to other cells against RVFV. We now wanted to see if endosomes played a role in the antiviral activity of PHTCM against RVFV in our experiments. To test the role of endosomes in

the ability of PHTCM to confer resistance we used sonication. Pretreating PHTCM with sonication destroys the exosomes present in the PHTCM and should interfere with its ability to confer resistance.

The results shown in Figure 7 demonstrate that sonicated PHTCM losses some of its ability to confer viral resistance to A549 cells but not significantly. The data can only be interpreted as suggestive here but it would seem to indicate that exosomes do play a role in conferring resistance against RVFV. The data also suggests that there are other components of the PHTCM that also play a limited role. It should be noted that in experiments preformed by Delorme-Axford et al., sonication completely abolished the antiviral effect of PHTCM against VSV [6]. The mechanism for the antiviral activity of PHTCM may be virus specific and in the case of RVFV it may not be mediated exclusively by the exosomes. The components of PHTCM require further investigation as to why they are so effective against a wide range of viruses and could be important in understanding why some diseases are vertically transmitted while others are not. PHTCM is clearly varies between donors and this could have important real world implications in the case of human vertical transmission events. The possibility exits that the differences between PHTCM samples we observed were the result of variables in the cell culturing process. Other reasons for variations between donors could have something to do with an individual's genetics or even nutrition level but regardless, they need to be investigated further.

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