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Zika Virus RNA Degradation In Municipal Wastewater

Aaron Muirhead

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By

AARON L. MUIRHEAD

INTRODUCTION: Zika virus (ZIKV) resurfaced in 2015 and caused a worldwide epidemic. ZIKV emerged as a potential cause of the serious birth defect of microcephaly, and the rare neurological disorder Guillain-Barre syndrome. An estimated 80% of ZIKV cases are asymptomatic, and treatment for the disease is not mandated until an infection of ZIKV is confirmed. This has caused a challenge in determining the true burden ZIKV has on communities that face epidemics. There has been a methodological and research gap in the detection and recovery of enveloped viruses, such as ZIKV, in environmental waters. Recent work has shown that ZIKV RNA is better detected in urine opposed to serum samples. This suggest that exploration of methods to understand the detectability and survivability of ZIKV in various waters and highlights the potential to detect it in municipal wastewater in low-resource settings.

AIM: The objectives of this study are to develop a system for laboratory study to detect ZIKV RNA in environmental waters and to evaluate the stability of ZIKV RNA in sewage under three relevant temperatures.

METHODS: ZIKV MEX 1-44 was added to primary effluent from a local municipal wastewater treatment plant. Two experiments were conducted. Experiment 1 was meant to assess the feasibility of detecting ZIKV RNA. Samples were held at 25°C and RNA was extracted at days 0, 1, and 7. Experiment 2 consisted of samples held at 4°C, 25°C, 35°C and RNA was extracted at days 0, 7, 14, and 29. Quantitative polymerase chain reaction (qPCR) was used to quantify ZKV RNA concentration. A general linear mixed model was applied to account for repeated measurements and compare groups to calculate the amount of time to virus inactivation.

RESULTS: ZIKV RNA was detected in all samples at all times and temperatures with qPCR. At the 3 temperatures, ZIKV RNA degradation occurred over the 29 days in the 25°C and 35°C temperature groups and was more rapid in the 35°C group. There was little to no decrease observed in ZIKV RNA at 4°C.

DISCUSSION: ZIKV RNA is more environmentally stable than assumed. This study was a pilot study to develop evidence for further exploration of materials and methods for detection of ZIKV RNA in wastewater. Cost-effective methods to detect ZIKV RNA in municipal wastewater such as this will be of crucial importance to better understand the burden ZIKV has on a community during epidemics due to challenges faced on detecting ZIKV infections in the clinical setting.

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AARON L. MUIRHEAD

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30303

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ZIKA VIRUS RNA DEGRADATION IN MUNICIPAL WASTEWATER

by

AARON L. MUIRHEAD

Approved:

Dr. Christine Stauber
Committee Chair

Dr. Matthew Hayat
Committee Member

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AUTHOR'S STATEMENT

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Author's Signature

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LIST OF ABBREVIATIONS

ZIKV – Zika virus

RNA – ribonucleic acid

qPCR – Quantitative Polymerase Chain Reaction

RT-PCR – Reverse Transcription Polymerase Chain Reaction

ICC - Intraclass Correlation Coefficient

INTRODUCTION

Zika virus (ZIKV) was first isolated in Uganda in 1947 [1], but it was not until 2007 a large-scale human outbreak of ZIKV was reported in Yap Island of the Federated States of Micronesia [2]. Outbreaks since have continued worldwide, many within the country of Brazil. The exact dissemination point of ZIKV human outbreaks in Brazil differs with some retrospective studies suggesting that ZIKV was circulating in southern Brazil from April to November 2013 [3-5], and others suggested the northeastern region of Brazil in February 2014 [6]. In March 2015, Brazil reported a novel outbreak to the World Health Organization [7] with the initial reports stating symptoms were of a mild severity and tests were negative for chikungunya, measles, rubella, parvovirus B19, and enterovirus [7]. ZIKV was not suspected at this stage, and no tests for ZIKV were carried out. In May 2015, Brazil confirmed ZIKV virus was circulating in the country [7] and was also later confirmed in Bahia, Brazil by the molecular method of reverse transcription polymerase chain reaction (RT-PCR) [8]. Over the next two years ZIKV emerged as a potential cause of the serious birth defect of microcephaly, and the rare neurological disorder Guillain-Barre syndrome. This disease in causes the body's immune system to accidentally attacks part of its peripheral nervous system [9, 10]. Brazil had a notable increase in cases of microcephaly in newborns the summer of 2015. In late 2015, Brazil confirmed that the increase was associated with ZIKV that had infected pregnant women [11].

Similar to the dengue and chikungunya viruses, ZIKV is primarily spread by the mosquito *Aedes aegypti,* [1]. This domestic mosquito resides largely in tropical and sub-tropical regions. Ecological spatial analysis revealed that the northeastern region of Brazil has the highest risk of *Aedes aegypti* house infestation due to the man-made habitats best suited for larval development [12]. Since the initial outbreak of ZIKV in Brazil in 2015, 84 countries have discovered evidence of ZIKV transmission [13]. As of 2017, 23 countries have had increases of

Guillain-Barre syndrome and ZKV infections; and 31 countries have had an increase in microcephaly which was likely associated to ZIKV infection [13, 14]. Added to the current global impact, the effects of climate change may cause the spread of ZIKV into new geographical regions in the future [15].

There has been a methodological gap in the detection and recovery of enveloped viruses in environmental waters. There is a long history of research that has focused on the detection and fate of non-enveloped enteric viruses in municipal wastewater and that they are mainly transmitted by the fecal-oral route (e.g., adenoviruses, polioviruses, enteroviruses, noroviruses and rotaviruses) [16-19]. Due to this focus, there are well-established methods for detecting non-enveloped viruses in wastewaters. Enveloped viruses are structured differently. Enveloped viruses have a lipid bilayer membrane that is outside of the viral protein capsid that are not present in their non-enveloped counterparts. This layer has proven to impact their survival in aquatic environments [20, 21]. This also means that methods developed to detect non-enveloped viruses in wastewaters may not be ideal for detecting enveloped viruses as well. It has been found that common organic solvents [22] used in the concentration and purification process for non-enveloped enteric viruses are not suitable for enveloped viruses due to the sensitivity of the lipid bilayer.

Based on the widely cited research that found ~80% of infected ZIKV patients to be asymptomatic [2], effective methods to detect ZIKV in sewage and environmental waters will be required to determine the burden on a community when it may not be properly assessed in resource poor settings. Currently, surveillance efforts by the Pan American Health Organization and World Health Organization mandate that only individuals that display ZIKV-like symptoms undergo laboratory testing for ZIKV infection [23]. This underestimates the true prevalence of

infection and related complications, leaving current estimations to be biased towards those who seek care or develop symptoms to an infection [24]. Due to these potential errors in determining true prevalence, coupled with evidence discovering that enveloped viruses are more stable in the environment than presumed [25], developing cost effective measures to detect ZIKV burden on a community has become more feasible.

Increasing the understanding of developing a cost-effective detection method for ZIKV and potentially other enveloped viruses will allow resource-limited settings to assess for disease burden during an epidemic. This study was done with following aims:

- To develop a system for laboratory study of ZIKV RNA in environmental waters
- To evaluate the stability of ZIKV RNA in sewage under three relevant temperatures

Literature Review

Envelope virus survival and degradation

ZIKV's lipid bilayer membrane outside the viral protein capsid contains glycoproteins, which makes it an enveloped virus [20]. The various functional groups on the outer surface of enveloped viruses compared to non-enveloped viruses, which does not have this outer membrane, likely limits enveloped virus' survival and partitioning behavior in liquid environments [21, 26]. Due to this impact on the survivability, more research is needed to understand the ability for enveloped viruses to exist in detectable concentrations outside of their hosts in the environment. In recent years, this research has begun to address the ability of enveloped viruses to withstand environments outside of the body. A recent review noted that the time for 90% inactivation varied from hours to months for avian influenza (an enveloped virus) under relevant environmental conditions; it lasted as long as some nonenveloped viruses in similar conditions [27]. A study that compared the concentration value of Phi6, a commonly used enveloped virus surrogate, to other enveloped viruses in numerous liquid mediums highlighted the variability of enveloped virus' persistence in the environment [25]. Under epidemic conditions, wastewater facilities must increase treatment methods to avoid transmitting enveloped viruses. Although treated wastewater does reduce viral load, enveloped viruses [10] and other infectious human viruses [28-30] have been found to be able to survive within wastewaters and have been detectable in wastewater treatment effluent. Also, the 2014 Ebola (an enveloped virus) cases in the U.S. exposed how little research has been conducted surrounding the presence and fate of enveloped viruses in human waste and sewage [31]. At the time, there were few studies that focused on the survivability of Ebola virus outside of the human body and few in municipal wastewater were conducted. Added to this, government agencies announced to the public that Ebola was not contagious outside of the body and wastewater treatment

procedures resulted in low risks of transmission. Since then, research has been initiated on the fate of Ebola virus and Ebola virus surrogates in the environment [32, 33], and research has found Ebola to have persisted in sterile wastewater for up to 8 days [31]. The assumption that Ebola rapidly inactivates outside of the human body still has not been validated.

Detection of enveloped virus

Detection of non-enveloped viruses in environmental waters has been widely studied due non-enveloped virus' survivability but developing detection methods for non-enveloped and enveloped viruses are not same. Methods to concentrate and recover enveloped and non-enveloped viruses differ and may not be suitable for one compared to the other. As previously mentioned, enveloped virus' lipid layers are sensitive to the organic solvents (i.e. sodium deoxycholate, and phospholipase A.) [22] that are commonly used to extract and purify non-enveloped enteric viruses. Recent work may have found a more suitable method by optimized ultrafiltration. This method yielded 18-25% recovery for two enveloped viruses in wastewater [10]. This research also tested a ultracentrifugation method to pellet wastewater solids, and concluded that the utilization of this method can effectively recover enveloped virus' from wastewater for qPCR detection [10].

Detecting ZIKV RNA in urine has increased in interest in recent years. Many studies describe virus detection that persists for longer durations in urine than in serum [34-38]. Work that compared test results for ZIKV RNA in different media found approximately twice as many persons with RT-PCR positive test results for ZIKV RNA in urine specimens, 61 persons (92%), compared with 31 persons (47%) from serum specimens respectively [39]. These studies reiterate that the notion that sampling urine opposed to serum can increase the number of

laboratory-confirmed cases in areas facing an epidemic because of a longer period of RNA detection, higher RNA levels, and less invasive sample collection [38].

In laboratory settings, the detection of ZIKV in urine can be affected by many factors including matrix-specific effects, handling, and storage of specimens. Little knowledge is known about the stability of ZIKV RNA in urine. RNA can be prone to rapid degradation in urine because urine is a suitable environment for a high activity of hydrolysis, up to 100 times higher than serum [40]. To counteract this, nucleic acid stabilizers must be used to minimize RNA degradation to slow the hydrolysis process and preserve the nucleic acids originally within the samples [41-43]. When stored at −80 °C, a common storage temperature, ZIKV RNA in urine samples suffered a significant loss of PCR detection without nucleic acid stabilizers [44]. At the same temperature, the addition of nucleic acid stabilizers to urine samples significantly corrected this effect and resulted in recovery of ZIKV RNA in all samples [44].

Wastewater epidemiology is a growing field that has had numerous studies centered on understanding the transmission and detection of viruses, with majority focusing on nonenveloped enteric viruses. With the increased interest and feasibility of studying detection of enveloped viruses in environmental water, employing detection methods suitable for ZIKV is a viable option. Considering ZKV is largely an asymptomatic disease added to the ability to detect ZKV in urine, this provides an opportune moment to employ research that develop detection methods for ZIKV which compensate for missing ZIKV cases in the clinical and expand the field of wastewater epidemiology.

Application of Multilevel modeling

Multilevel modeling is an analytical approach that is an appropriate modeling framework for repeated measures data. Multilevel modeling can account for the within and between subject relationship for an outcome in a repeated measures setting [45].

With the collection of longitudinal data that is formed by repeated measurements on a sample or individual, a general linear mixed model is a type of multilevel model that accounts for within subject correlation. The within subject correlation for repeated measurements taken over time can be accounted for with a random effect [46]. Group effects over time can be compared with a group x time interaction term [46]. Mixed models that include random effects (random effects model) provides the opportunity to analyze the variance of the dependent variable based on within- and between- group components. Then, the proportion of total variance due to between-subject variance is quantified using the intraclass correlation coefficient (ICC) [47].

Multilevel modeling has been applied to many aspects of epidemiology (9-11) and sexually transmitted infections (5-8) research in recent years due to its many advantages of determining effects on individuals effected, the communities they are located in, and the geographical location of the community. In the medical field, multilevel modeling is applied to medical research for patients nested within a physician's care, departments, and hospitals. Multilevel modeling can also be applied to microbiological experiments that have multiple experimental variables as well as repeated measures over time.

Materials and Methods

ZIKV MEX 1-44 was grown in mosquito cells to a concentration of 10^6 plaque forming units (pfu) per ml in the laboratory of Dr. Margo Brinton. The culture was stored at −80 °C until use. Primary effluent was collected from a local wastewater treatment plant in 1-liter plastic bottles and stored at 4ºC prior to use. The effluent was stored up to 7 days prior to inoculation with ZIKV.

Controlled laboratory experiments

We performed two laboratory experiments to examine the persistence of ZIKV RNA in untreated primary effluent: one at room temperature (~25℃) and one at three controlled temperature treatment groups: 4℃, 25℃, 35℃. In the both experiments in triplicate, we spiked ZIKV MEX 1-44 into each sample to achieve a concentration of 10^5 pfu/ml in unpasteurized primary effluent. In experiment 1, the ZIKV spiked samples were stored at room temperature ~25ºC for a period of 7 days. At day 0, 1, 3, and 7, we removed aliquots and extracted nucleic acids. In experiment 2, the samples were stored in incubators or the refrigerator at 4 ºC, 25 ºC, and 35 ºC. During experiment 2, we extracted nucleic acid from the samples at days 0, 7, 14, and 29. During both experiments, primary effluent that was not spiked with ZIKV was also incubated and extracted to act as a biological control.

Nucleic acid extraction was performed using the QiaAMP Minelute Virus Spin Kit per kit instructions. All extractions were carried out according to the manufacturer's recommendations and were eluted to a final volume of 50 µL containing Buffer AVE. All samples were aliquoted into duplicate samples of 25 µL and stored in -20 ºC until qPCR was performed.

Samples were tested for ZIKV RNA using one-step real-time reverse transcription polymerase chain reaction (RT-RNA) assay for the qualitative detection of RNA via the

TaqPath™ Zika Virus Kit (Fisher Scientific). RT-PCR was performed on Applied Biosystems™ 7500 Real-Time PCR Systems Instrument and involved 25 µL reactions of the RT-PCR TaqPath™ Zika Virus Kit and 2.5 µL of RNA extract. Cycling conditions were based on the recommendations of the manufacturer and are listed in table 2. In addition to the spiked and biological controls, a known concentration of ZIKV RNA was also included in the reactions to act as a standard curve. Each run included a negative and positive controls for ZIKV. For quantification, standard curves were prepared containing the target sequences with the assumption that the ATCC RNA standard was at 10^5 copy number / µL.

Data Analysis

For experiment 2, each replicate (A, B, C) that was in each temperature condition (4°C, 25°C, 35°C) was extracted at 4 time points (day 0, 7, 14, 29) for a total of 36 repeated measures. For the statistical analysis, qPCR results from experiment 2 was used and a general linear mixed model applied. Treatment group of 4°C was used as the reference category in the model. Group by time interaction terms were included in the model to quantify comparative change. The intraclass correlation coefficient was calculated. Analysis was completed in SAS 9.4.

Data from experiment 2 were used to fit a line for each temperature in Microsoft Excel Version 16.6.4. The equations were then used to calculate the amount of time (days) for $log_{10}\left[\frac{C_t}{C_0}\right]$ reductions to occur at 90% (-1 $log_{10}\left[\frac{C_t}{C_0}\right]$), 99% (-2 $log_{10}\left[\frac{C_t}{C_0}\right]$), 99.9% ($log_{10}\left[\frac{C_t}{C_0}\right]$).

Results

ZIKV RNA degradation for both experiments were assessed by qPCR. Both experiments differed by the temperatures the samples were exposed to. The qPCR run for experiment 1 tested samples that were held at a constant temperature (25℃) and had ZIKV RNA extracted on days 0, 1, 3, and 7. Although all samples were extracted, only days 0, 1 and 7 were analyzed via qPCR because of time and material constraints. In experiment 2, the samples were analyzed for all conditions and all extractions. The qPCR run for experiment 2 tested samples that were exposed to three relevant temperatures (4℃, 25℃, 35℃) and had ZIKV RNA extracted on days 0, 7, 14, and 29.

The results of the initial qPCR experiment are displayed in Figure 1. As shown in figure 1, the qPCR cycle on the X-axis and the RNA concentration on the y-axis. In addition to the sample replicates, the data are also provided for the standard curve. As shown by the pink, blue, and green lines, ZIKV RNA was detectable for all conditions initiating at cycle 18. This corresponds to a concentration (compared to the standard) of at least 10^5 RNA copies or higher. Also notable in figure 1, concentrations as low as 10 or 1 RNA copy were detectable via this assay.

The remaining results only displayed the calculated concentrations of ZIKV RNA copies. Figures 2 and 3 display the ZIKV RNA log_{10} concentration quantity and $log_{10}\ [\frac{C_t}{C_0}]$ of experiment 1 under constant temperature. Figures 3 and 4 display the ZIKV RNA log_{10} concentration quantity and $log_{10}\ [\frac{C_t}{C_0}]$ of experiment 2 under the three temperatures.

As shown in Figures 2 and 3, the initial concentration of ZIKV RNA measured via qPCR assay indicated higher than inoculated concentrations (estimated to be approximately 10^9 copies) for time point 0 for rep A and B. This decreased almost three-fold on day 1 and was closer to the

concentration expected. This suggests that the initial concentration measurement was in error for day 0 for A. When comparing day 1 and day 7, ZIKV RNA concentration did not decrease greatly over the time when stored at room temperature (both days average concentration were 10^5 copies).

In the second experiment, ZIKV RNA degradation demonstrated a steady decline at 25°C, going from 10^5 copies on day 0 to 10^3 copies on week 4. At 35°C, there as a more rapid decline in ZIKV RNA degradation with 10^5 copies on day 0, to 10^2 copies on day 14, to the to 10^1 copies at day 29. At 4°C, on average, there was a slight increase in ZIKV RNA quantity from baseline to slightly above baselines concentrations at day 14 in all three replicates, and degradation was not detected until day 29 (Figure 4 and 5).

Regression coefficients were calculated within a linear regression model with mixed effects. Interaction variables were added to the model to describe the comparative change in the response of interest for the groups. Analysis revealed that interaction variables for time x temperature groups 25°C and 35°C were significantly different ($p < 0.05$) than the time x temperature group at 4°C (Table 3). The ICC was calculated to describe how much the data in the same group resembles each other. With a low ICC of 0.112 and the value being closer to zero, we can conclude that data in the same groups are not similar (Table 5).

Regression lines were calculated in Microsoft Excel to determine $log_{10}\left[\frac{C_t}{C_0}\right]$ reductions for the three temperature groups. As shown in table 4, the predicted values calculated from the regression lines to achieve 90% ($-1\ log_{10}$), 99% ($-2\ log_{10}$), and 99.9% ($-3\ log_{10}$) reduction of ZIKV at 4°C, 25°C, and 35°C. Based on the regression line, at 4°C, it would take 123 days for a 90% reduction, 209 days for a 99% reduction, and 295 days for 99.9% reduction for ZIKV RNA degradation. At 25°C, it would take 14 days for a 90% reduction, 28 days for a 99% reduction,

and 41 days for 99.9% reduction for ZIKV inactivation. At 35°C, it would take 6 days for a 90% reduction, 13 days for a 99% reduction, and 20 days for 99.9% reduction for ZIKV inactivation (Table 4).

Discussion of Research Questions

ZIKV is a mosquito-borne enveloped virus that rapidly spread throughout the Americas and Caribbean in 2015. Health complications from ZIKV reached epidemic proportions in 2016. This lead to the WHO to declare a public health emergency of global concern in the same year [7]. Currently, ZIKV is primarily spread by *Aedes* mosquitoes, particularly *Aedes aegypti* and *Aedes albopictus* [48]. Recent research has created a global map of locations that are suitable for seasonal and year-round presence of *Aedes* mosquitoes, revealing that the year-round presence are locations closest to the Equator, and seasonal presence covering a significantly larger area [49]. These locations that face a year-round presence are a higher potential risk of the transmission of ZIKV, and may benefit the most from a cost-effective detection method of ZIKV. With an estimated 80% of ZIKV infections to be asymptomatic [2, 50], and symptoms that do appear are similar to many other diseases [51], other detection methods must be implemented in settings that face ZIKV endemics.

The diagnosis of ZIKV infection has relied heavily on the detection of ZIKV RNA or ZIKV antibodies in serum (mostly in the clinical setting). This route has been found to be challenging, with the detection duration of ZIKV in serum to be short lived and the possibility of cross reactivity of ZIKV antibodies to other diseases such as dengue virus. Many reports have shown that the detection of ZIKV in urine is longer than serum [34-38]. Little to no research has performed on the survivability of ZIKV in waters or wastewater. Wastewater epidemiology is a growing field, and this study adds to our understanding the potential to detect ZIKV in wastewater. Similar to the work of Ye et al. that used model enveloped viruses, this study was

able to detect ZIKV RNA, an enveloped virus, in spiked, untreated wastewater over weeks at various conditions. A recent meta-analysis has found that envelope viruses can take hours and up to weeks for a 90% virus reduction to occur in relevant environmental conditions [25].

In this study, there was a detected increase in ZIKV RNA concentration within each replicate at 4°C at time point 0 and 7 until day 14 in Experiment 2. There was an approximate 10-fold higher concentration in the samples held at 4°C compared to 25°C. Similar results were found when ZIKV RNA concentration was quantified to understand ZIKV stability in urine. Within the first 48 hours, while stored at 4°C, there was little decrease in ZIKV RNA concentration and concentrations were higher at each time point compared to samples held at 25°C [44].

Study Strengths and Limitations

The strengths of this study include the use of unpasteurized primary effluent and multiple temperatures. This is the first study to evaluate the persistence of ZIKV RNA outside of the clinical conditions and setting. We document ZIKV RNA persistence for weeks at high temperatures suggesting that the ZIKV may be detectable under environmental conditions found in many endemic settings.

The results of this study adds to the growing field of wastewater epidemiology. The assumption that enveloped viruses rapidly inactivate outside of the human body and in aqueous environment continues are in contrast with our findings. Utilizing wastewater samples can also be a valuable alternative or addition to ZIKV infection data collected in the clinical setting.

There are three main limitations of this study. First, the unpastuerized wastewater was not sourced from a location that has been exposed to a known ZIKV endemic. Second, the results of this study cannot conclude that the detection of ZIKV RNA is associated with infectivity of

ZIKV. Third, the quantification of ZIKV RNA concentration using qPCR is not the most ideal method of quantification as the intention of the kit was for qualitative detection.

Recommendations of Future Findings

Future work can be done to further document the ZIKV infectivity to determine if the virus is intact or if only the RNA persisted. This can also inform collection of wastewater samples to further the knowledge on the detection and survivability of enveloped viruses for the field of wastewater epidemiology. The application of this procedure with sewage samples from endemic areas can be an area of future research for research and clinical purposes. Research can also focus on the likelihood of infectivity of ZIKV from the detection of ZIKV RNA.

References

1. Dick, G.W., S.F. Kitchen, and A.J. Haddow, *Zika virus. I. Isolations and serological specificity.* Trans R Soc Trop Med Hyg, 1952. **46**(5): p. 509-20.
2. Duffy, M.R., et al., *Zika virus outbreak on Yap Island, Federated States of Micronesia.* N Engl J Med, 2009. **360**(24): p. 2536-43.
3. Metsky, H.C., et al., *Zika virus evolution and spread in the Americas.* Nature, 2017. **546**(7658): p. 411-415.
4. Ayllon, T., et al., *Early Evidence for Zika Virus Circulation among Aedes aegypti Mosquitoes, Rio de Janeiro, Brazil.* Emerg Infect Dis, 2017. **23**(8): p. 1411-1412.
5. Passos, S.R.L., et al., *Detection of Zika Virus in April 2013 Patient Samples, Rio de Janeiro, Brazil.* Emerg Infect Dis, 2017. **23**(12): p. 2120-2121.
6. Faria, N.R., et al., *Establishment and cryptic transmission of Zika virus in Brazil and the Americas.* Nature, 2017. **546**(7658): p. 406-410.
7. Organization, W.H. *The History of Zika Virus* 2016 [cited 2019; Available from: https://www.who.int/emergencies/zika-virus/timeline/en/.
8. Campos, G.S., A.C. Bandeira, and S.I. Sardi, *Zika Virus Outbreak, Bahia, Brazil.* Emerg Infect Dis, 2015. **21**(10): p. 1885-6.
9. Mlera, L., W. Melik, and M.E. Bloom, *The role of viral persistence in flavivirus biology.* Pathog Dis, 2014. **71**(2): p. 137-63.
10. Ye, Y., et al., *Survivability, Partitioning, and Recovery of Enveloped Viruses in Untreated Municipal Wastewater.* Environ Sci Technol, 2016. **50**(10): p. 5077-85.
11. de Araujo, T.V.B.X., R. A. A.; Miranda-Filho, D. B.; Souza, W. V.; Montarroyos, U. R.; de Melo, A. P. L.; Valongueiro, S.; de Albuquerque, M.; Braga, C.; Filho, S. P. B.; Cordeiro, M. T.; Vazquez, E.; Cruz, D.; Henriques, C. M. P.; Bezerra, L. C. A.; Castanha, P.; Dhalia, R.; Marques-Junior, E. T. A.; Martelli, C. M. T.; Rodrigues, L. C.; investigators from the Microcephaly Epidemic Research, G.; Brazilian Ministry of, H.; Pan American Health, O.; Instituto de Medicina Integral Professor Fernando, F.; State Health Department of, P., *Association between microcephaly, Zika virus infection, and other risk factors in Brazil: final report of a case- control study.* The Lancet Infectious Disease, 2018. **18**(3): p. 328-336.
12. Campos, M.C., et al., *Zika might not be acting alone: Using an ecological study approach to investigate potential co-acting risk factors for an unusual pattern of microcephaly in Brazil.* PLoS One, 2018. **13**(8): p. e0201452.
13. Organization, W.H. *Situation report: Zika virus, microcephaly, Guillain-Barré syndrome*. 2016; Available from: http://www.who.int/iris/handle/10665/251462.
14. Sperling, M.v. *Urban Wastewater Treatment in Brazil*. August 2016 [cited 2019; Available from: https://publications.iadb.org/en/publication/12515/urban-wastewater-treatment-brazil.
15. Altizer, S., et al., *Climate change and infectious diseases: from evidence to a predictive framework.* Science, 2013. **341**(6145): p. 514-9.
16. Puig, M., et al., *Detection of adenoviruses and enteroviruses in polluted waters by nested PCR amplification.* Appl Environ Microbiol, 1994. **60**(8): p. 2963-70.
17. Katayama, H., et al., *One-year monthly quantitative survey of noroviruses, enteroviruses, and adenoviruses in wastewater collected from six plants in Japan.* Water Res, 2008. **42**(6-7): p. 1441-8.
18. Fumian, T.M., et al., *Detection of rotavirus A in sewage samples using multiplex qPCR and an evaluation of the ultracentrifugation and adsorption-elution methods for virus concentration.* J Virol Methods, 2010. **170**(1-2): p. 42-6.

19. Tambini, G., et al., *Direct detection of wild poliovirus circulation by stool surveys of healthy children and analysis of community wastewater.* J Infect Dis, 1993. **168**(6): p. 1510-4.
20. Arbely, E., et al., *A trimerizing GxxxG motif is uniquely inserted in the severe acute respiratory syndrome (SARS) coronavirus spike protein transmembrane domain.* Biochemistry, 2006. **45**(38): p. 11349-56.
21. Shigematsu, S., et al., *Influenza A virus survival in water is influenced by the origin species of the host cell.* Influenza Other Respir Viruses, 2014. **8**(1): p. 123-30.
22. Vidaver, A.K., R.K. Koski, and J.L. Van Etten, *Bacteriophage phi6: a Lipid-Containing Virus of Pseudomonas phaseolicola.* J Virol, 1973. **11**(5): p. 799-805.
23. Organization, P.A.H. *Guidelines for surveillance of Zika virus disease and its complications*. 2016; Available from: http://iris.paho.org/xmlui/handle/123456789/28405.
24. Lozier, M., et al., *Incidence of Zika Virus Disease by Age and Sex - Puerto Rico, November 1, 2015-October 20, 2016.* MMWR Morb Mortal Wkly Rep, 2016. **65**(44): p. 1219-1223.
25. Aquino de Carvalho, N., et al., *Evaluation of Phi6 Persistence and Suitability as an Enveloped Virus Surrogate.* Environmental Science & Technology, Aug 2017. **51**: p. 8692-8700.
26. Gundy, P.M., Gerba, C.P. & Pepper, I.L., *Survival of Coronaviruses in Water and Wastewater.* Food and Environmental Virology, 2009(1:10): p. 10-14.
27. Wigginton, K.R., Ye, Y., Ellenberg, R.M., *Emerging investigators series: the source and fate of pandemic viruses in the urban water cycle.* Environmental Science: Water Research & Technology, 2015(6): p. 735-746.
28. Hot, D., et al., *Detection of somatic phages, infectious enteroviruses and enterovirus genomes as indicators of human enteric viral pollution in surface water.* Water Res, 2003. **37**(19): p. 4703-10.
29. Hewitt, J., et al., *Influence of wastewater treatment process and the population size on human virus profiles in wastewater.* Water Res, 2011. **45**(18): p. 6267-76.
30. Petrinca, A.R., et al., *Presence and environmental circulation of enteric viruses in three different wastewater treatment plants.* J Appl Microbiol, 2009. **106**(5): p. 1608-17.
31. Bibby, K., et al., *Persistence of Ebola Virus in Sterilized Wastewater.* Environ Sci Technol Lett, 2015. **2**(9): p. 245-249.
32. Casanova, L.M. and S.R. Weaver, *Evaluation of eluents for the recovery of an enveloped virus from hands by whole-hand sampling.* J Appl Microbiol, 2015. **118**(5): p. 1210-6.
33. Cook, B.W., et al., *Evaluating environmental persistence and disinfection of the Ebola virus Makona variant.* Viruses, 2015. **7**(4): p. 1975-86.
34. Barzon, L., et al., *Isolation of infectious Zika virus from saliva and prolonged viral RNA shedding in a traveller returning from the Dominican Republic to Italy, January 2016.* Euro Surveill, 2016. **21**(10): p. 30159.
35. Fourcade, C., et al., *Viral load kinetics of Zika virus in plasma, urine and saliva in a couple returning from Martinique, French West Indies.* J Clin Virol, 2016. **82**: p. 1-4.
36. Gourinat, A.C., et al., *Detection of Zika virus in urine.* Emerg Infect Dis, 2015. **21**(1): p. 84-6.
37. Zhang, F.C., et al., *Excretion of infectious Zika virus in urine.* Lancet Infect Dis, 2016. **16**(6): p. 641-642.
38. Campos Rde, M., et al., *Prolonged detection of Zika virus RNA in urine samples during the ongoing Zika virus epidemic in Brazil.* J Clin Virol, 2016. **77**: p. 69-70.
39. Bingham, A.M., et al., *Comparison of Test Results for Zika Virus RNA in Urine, Serum, and Saliva Specimens from Persons with Travel-Associated Zika Virus Disease - Florida, 2016.* MMWR Morb Mortal Wkly Rep, 2016. **65**(18): p. 475-8.
40. Bryzgunova, O.E. and P.P. Laktionov, *Extracellular Nucleic Acids in Urine: Sources, Structure, Diagnostic Potential.* Acta Naturae, 2015. **7**(3): p. 48-54.

41. Anwar, A., et al., *Evaluation of pre-analytical variables in the quantification of dengue virus by real-time polymerase chain reaction.* J Mol Diagn, 2009. **11**(6): p. 537-42.
42. Blow, J.A., et al., *Viral nucleic acid stabilization by RNA extraction reagent.* J Virol Methods, 2008. **150**(1-2): p. 41-4.
43. Medeiros, M., et al., *Optimization of RNA yield, purity and mRNA copy number by treatment of urine cell pellets with RNAlater.* J Immunol Methods, 2003. **279**(1-2): p. 135-42.
44. Tan, S.K., et al., *Stability of Zika virus in urine: Specimen processing considerations and implications for the detection of RNA targets in urine.* J Virol Methods, 2017. **248**: p. 66-70.
45. Diez Roux, A.V. and A.E. Aiello, *Multilevel analysis of infectious diseases.* J Infect Dis, 2005. **191 Suppl 1**: p. S25-33.
46. Hayat, M.J. and H. Hedlin, *Modern statistical modeling approaches for analyzing repeated-measures data.* Nurs Res, 2012. **61**(3): p. 188-94.
47. Rabe-Hesketh, S., Skrondal, A., *multilevel and longitudinal modeling using stata*. 2005, College Station, TX: Stata Press.
48. Chouin-Carneiro, T., et al., *Differential Susceptibilities of Aedes aegypti and Aedes albopictus from the Americas to Zika Virus.* PLoS Negl Trop Dis, 2016. **10**(3): p. e0004543.
49. Attaway, D.F., et al., *Zika virus: Endemic and epidemic ranges of Aedes mosquito transmission.* J Infect Public Health, 2017. **10**(1): p. 120-123.
50. Hennessey, M., M. Fischer, and J.E. Staples, *Zika Virus Spreads to New Areas - Region of the Americas, May 2015-January 2016.* MMWR Morb Mortal Wkly Rep, 2016. **65**(3): p. 55-8.
51. Rabe, I.B., et al., *Interim Guidance for Interpretation of Zika Virus Antibody Test Results.* MMWR Morb Mortal Wkly Rep, 2016. **65**(21): p. 543-6.

Table 1. Sample conditions for RNA extraction.

Experiment	Temperature	Replicate	Time of Extractions (days)
1	~25℃	A, B, C	0, 1, 3, 7
2	4℃ 25℃ 35℃	A, B, C A, B, C A, B, C	0, 7, 14, 29

Table 2. Details of the steps and thermal cycling conditions of the samples during qPCR.

Steps	**Thermal Cycling Conditions**	
Transcription	HOLD	50℃ for 10 min
Activation	HOLD	95℃ for 2 min
PCR Amplification	40 cycles	95℃ for 3 sec
		60℃ for 30 sec

Table 3. Parameter estimates for the general linear mixed model (*p<0.05)

Effect	Temp	Estimate (95% CI)	DF	p-value
Intercept	-	0.3444 (-0.7732, 1.4621)	2	0.316
Time	-	-0.0079 (-0.03728, 0.02132)	28	0.5814
Temp	4°C	0	Ref	Ref
Temp	25°C	-0.3441 (-1.027, 0.3387)	28	0.3108
Temp	35°C	-0.6754 (-1.3583, 0.0075)	28	0.0524
Time*Temp	4°C	0	Ref	Ref
Time*Temp	25°C	-0.0657 (-0.1072, -0.02428)	28	0.003*
Time*Temp	35°C	-0.1554 (-0.1968, -0.1139)	28	<.0001*

Table 4. Predicted times (in days) for log_{10} reductions for virus inactivation at three temperature groups

Temp	Reduction $log_{10}\left[\frac{C_t}{C_0}\right]$	Time* (days)
4°C	-1 (90%)	123
	-2 (99%)	209
	-3 (99.9%)	295
25°C	-1 (90%)	14
	-2 (99%)	28
	-3 (99.9%)	41
35°C	-1 (90%)	6
	-2 (99%)	13
	-3 (99.9%)	20

Table 5. Variance Estimates and Intracluster Correlation Coefficient

Variance Estimates		
Covariance Parameters	Subject	Estimate
Intercept	Replicate	0.03572
Residual	---	0.283
$Estimate\ of\ ICC = \frac{0.03572}{0.03572 + 0.283} = 0.112$		

Figure 1: qPCR results for the detection of ZIKV RNA from spiked sewage that was held at 25°C and extracted at day 0, 1 and 7.

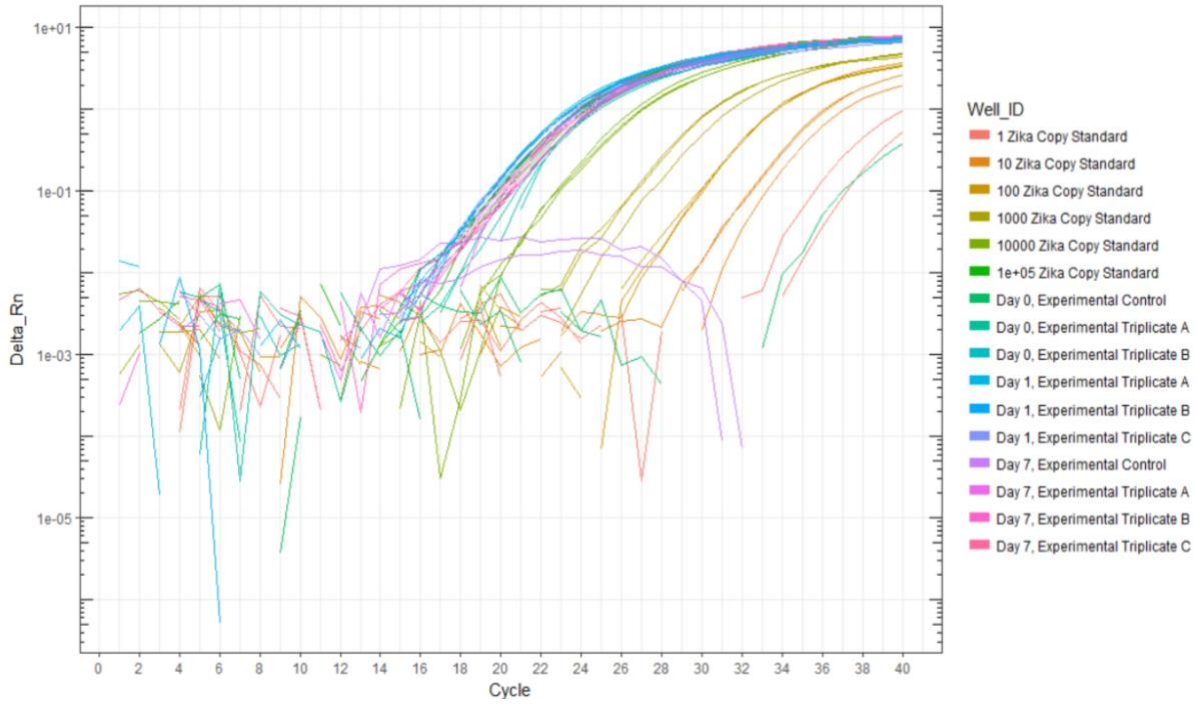


Figure 2. log_{10} concentration values of ZIKV RNA degradation at constant temperature of 25°C

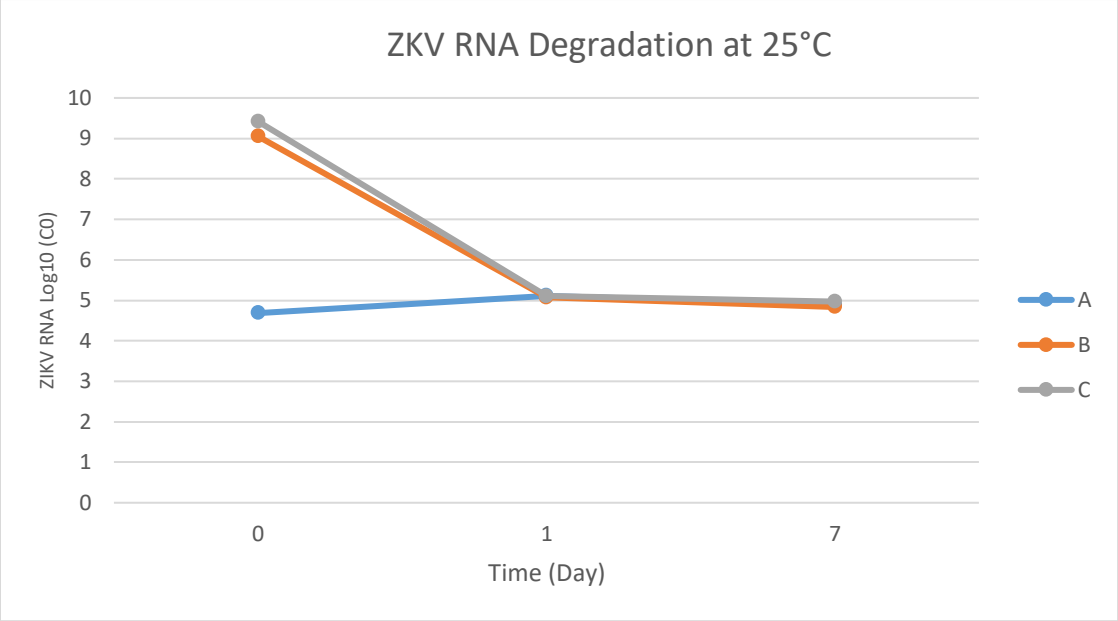


Figure 3. $log_{10}\left[\frac{C_t}{C_0}\right]$ values of ZIKV RNA degradation at constant temperature of 25°C

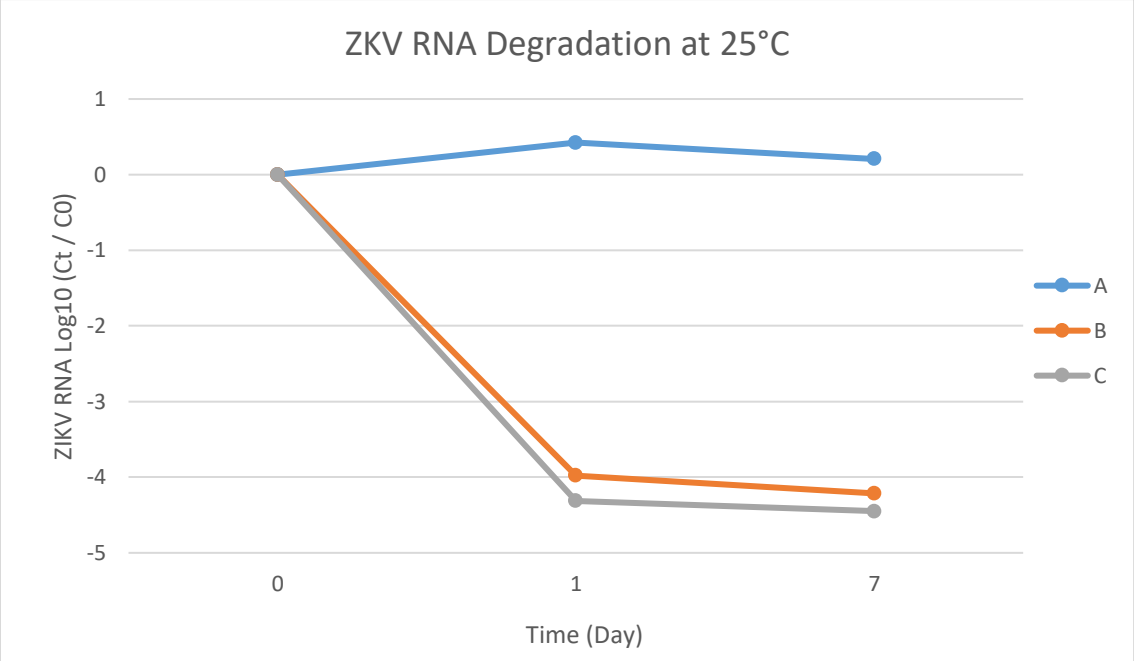


Figure 4. log_{10} concentration values of ZIKV RNA degradation at various temperatures of 4°C, 25°C, 35°C

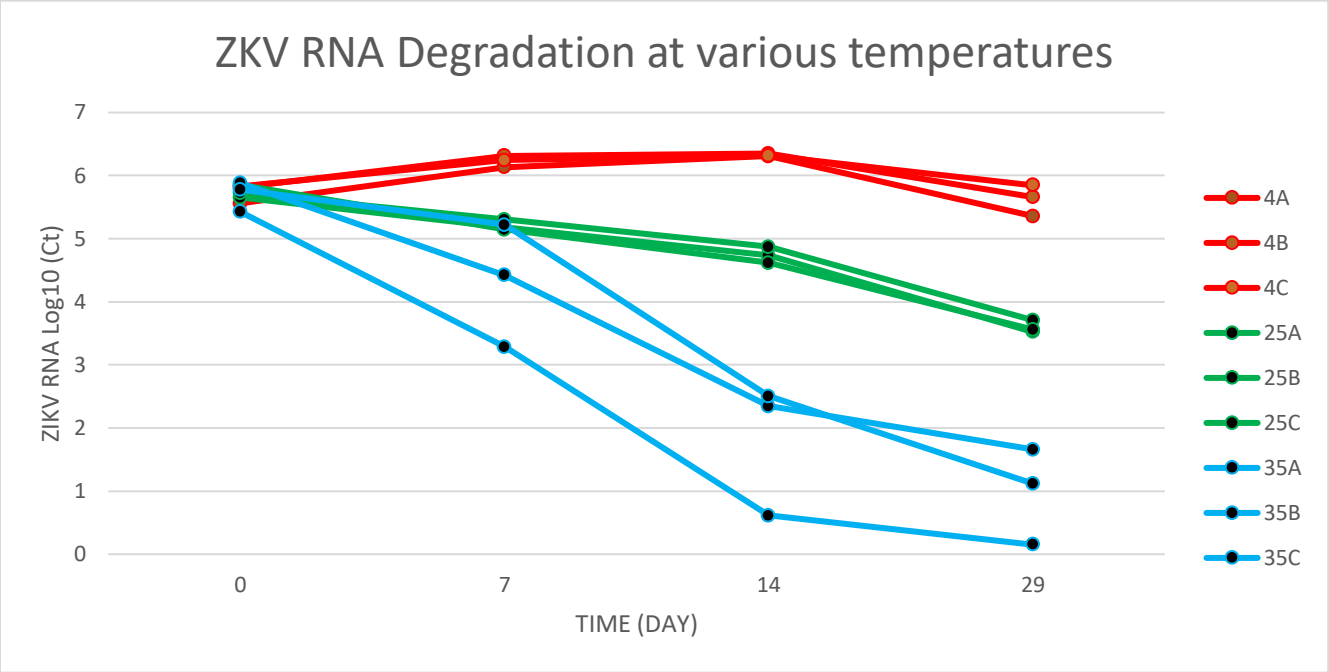
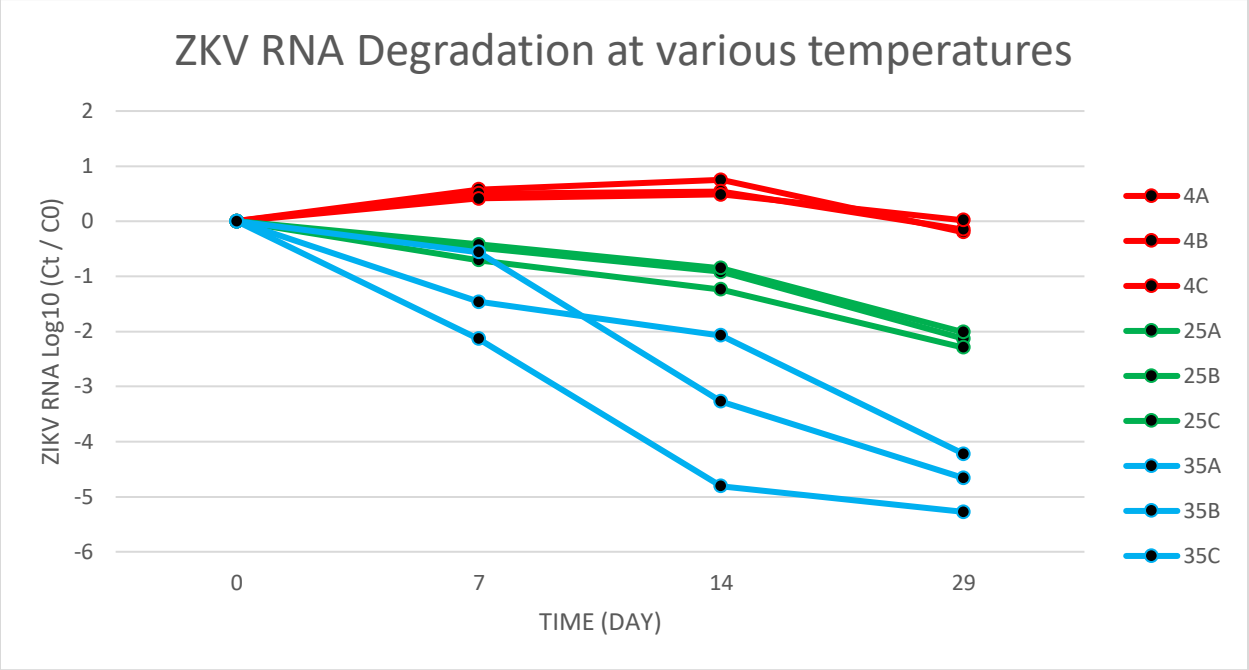


Figure 5. $log_{10}\left[\frac{C_t}{C_0}\right]$ values of ZIKV RNA degradation at various temperatures of 4°C, 25°C, 35°C



Appendix

SAS 9.4 Input Code for Mixed Model Analysis

```
data thesis2;
input temp $ time rep $ y;
datalines;
4 0 A 0
4 7 A 0.575641
4 14 A 0.751433
4 29 A -0.19964
4 0 B 0
4 7 B 0.502817
4 14 B 0.537913
4 29 B -0.15201
4 0 C 0
4 7 C 0.413423
4 14 C 0.487973
4 29 C 0.018496
25 0 A 0
25 7 A -0.47481
25 14 A -0.91801
25 29 A -2.13049
25 0 B 0
25 7 B -0.41935
25 14 B -0.85398
25 29 B -2.01454
25 0 C 0
25 7 C -0.70911
25 14 C -1.23836
25 29 C -2.29359
35 0 A 0
35 7 A -1.46241
35 14 A -2.07631
35 29 A -4.22073
35 0 B 0
35 7 B -0.55926
35 14 B -3.2746
35 29 B -4.65993
35 0 C 0
35 7 C -2.13746
35 14 C -4.81133
35 29 C -5.27542

;
run;
```

```
proc mixed data=thesis2;
class temp rep ;
model y = time temp time*temp / s CL;
random intercept / subject = rep s;
run;
```