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Laboratory and Epidemiological Characteristics of Zika Virus Infections in Florida

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LABORATORY AND EPIDEMIOLOGICAL CHARACTERISTICS OF ZIKA VIRUS INFECTIONS IN FLORIDA

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We have read this dissertation and recommend that it be approved.

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DEDICATION

First and foremost, this dissertation is dedicated to my wife, Kirsten, without whom this endeavor would have been impossible. Her love, support, encouragement, and patience was boundless, although I think we were about to test the limits on this… In my absence from home due to school and work, she continued to raise our children while teaching fulltime. She didn't even hesitate at letting me to drop pretty much everything and fly to Liberia during the Ebola outbreak in West Africa- less than six months after giving birth to Daughter #2. Six months later, all four of us were on a plane, moving to Liberia. My daughters, Kylie and Samantha, have also supported me with their love, even though I spend more time away from them than with them. Their support has enabled me to practice my craft and help countless others during the Ebola, Zika, and COVID-19 outbreaks. Without them, none of this would have been possible. This would have also been impossible without the support from my parents, Dohn and Lyn, who probably never envisioned the word 'doctor' next to my name. Lastly, this work is dedicated to my staff and those that have worked the bench alongside me and the unsung heroes of public health. Very truly, this work could not have been accomplished without those that worked tirelessly, day and night, to control the outbreak of Zika in Miami-Dade County and around the state. Their efforts conducting surveys, calling contacts, passing out mosquito repellant, shipping out specimens, working with the community, testing specimens, and the many, many other facets of public health, kept Zika at bay within our community and is truly something to celebrate.

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ABSTRACT OF THE DISSERTATION

LABORATORY AND EPIDEMIOLOGICAL CHARACTERISTICS OF ZIKA VIRUS INFECTIONS IN FLORIDA

by

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Until recently, Zika virus (ZIKV) was an obscure virus that rarely caused infections and was unknown to most. In 2015 and 2016, ZIKV came into the public spotlight as Brazil and other countries began to report large increases in infections with ZIKV and reported potential complications with developing fetuses and neurologic manifestations. In 2016, the state of Florida identified and responded to an outbreak of locally acquired ZIKV infections in Miami-Dade County. This dramatic increase in infections demonstrated both its importance as an emerging infectious disease and the paucity of knowledge surrounding ZIKV. This study seeks to utilize the data collected during the ZIKV pandemic to further characterize the virus and examine the efficacy of current diagnostic algorithms.

First, a systematic review was conducted to pool data from the literature on existing cases of ZIKV infections. Markov chain Monte Carlo modeling was used to determine a median incubation time of 6.5 days for infections with ZIKV. Median time to viral RNA clearance varied significantly by specimen type. Vaginal specimens

demonstrated the shortest time to viral RNA clearance (9.9 days); whereas blood specimens exhibited the longest (49.2 days).

Second, specimens from 934 symptomatic, non-congenitally acquired cases of ZIKV infection were analyzed to identify factors that contribute to the progression of viral load, as represented by the detection of ZIKV RNA. ZIKV RNA was detected most often in urine specimens and also was found to have higher viral loads than serum and whole blood specimens. Viral load was observed to be lower in non-pregnant women than pregnant women.

Last, an evaluation of the Centers for Disease Control and Prevention's (CDC) 2017 and 2019 ZIKV testing algorithms was conducted using data from all confirmed and probable cases identified in Florida between 2016 and 2018 (n = 1,522). ZIKV RNA was detected most frequently in urine specimens. When testing required plaque reduction neutralization test (PRNT) to discern between ZIKV and dengue virus, the PRNT assay was only able to discriminate between viruses about half of the time. Reducing the specimen collection window in the 2019 CDC algorithm resulted in fewer conclusive results.

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ABBREVIATIONS AND ACRONYMNS

aOR	Adjusted odds ratio
BPHL	Bureau of Public Health Laboratories
BOE	Bureau of Epidemiology
CDC	Centers for Disease Control and Prevention
cDNA	Complimentary deoxyribonucleic acid
CI	Confidence interval
CINAHL	Current Nursing and Allied Health Literature
CrI	Credible interval
CSF	Cerebrospinal fluid
C_t	Cycle threshold
DCPSO	Days collected post-symptom onset
DOH	Department of Health
DENV	Dengue virus
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunoassay
EMBASE	Excerpta Medica dataBASE
FIU	Florida International University
GBS	Guillain-Barré Syndrome
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IQR	Interquartile range

LDT	Laboratory-developed test
LILACS	Litertura Latino-Americana e do Caribe em Ciêncis de Saúde
LIS	Laboratory information system
MCMC	Markov chain Monte Carlo
MeSH	Medical subject heading
MEDLINE	Medical Literature Analysis and Retrieval System Online
MOH	Ministry of Health
NAAT	Nucleic acid amplification test
NPS	Non-pregnant symptomatic
OR	Odds ratio
PA	Pregnant asymptomatic
PAHO	Pan American Health Organization
PHEIC	Public Health Emergency of International Concern
PRNT	Plaque reduction neutralization test
PS	Pregnant symptomatic
RNA	Ribonucleic acide
RT-PCR	Reverse-transcriptase polymerase chain reaction
rRT-PCR	Real-time reverse-transcriptase polymerase chain reaction
WNV	West Nile virus
WHO	World Health Organization
YFV	Yellow fever virus
ZIKV	Zika virus

Chapter 1

Introduction

Background

The Zika virus (ZIKV) is an RNA virus that belongs to the *Flaviviridae* family, *Flavivirus* genus (1). Most *Flavivirus* species, including ZIKV, are transmitted through mosquito or tick vectors. Of note, several other clinically important species of viruses, including yellow fever virus (YFV), dengue virus (DENV), West Nile virus (WNV), and St. Louis encephalitis virus, share the same genus. The ZIKV was first isolated in 1947 in Uganda (2) and has since spread globally, causing an international outbreak in 2015 and 2016.

Between its discovery in 1947 and 2007, only 14 cases of ZIKV infection in humans were reported (3). Several serosurveys that indicated the potential for endemicity of ZIKV in many African and Asian countries were conducted in the latter half of the 20th century. However, results of these surveys must be interpreted with caution due to incomplete data, variation in laboratory methods, and the high rate of cross-reactivity among the different species of the *Flavivirus* genus (4).

In 2007, an outbreak of ZIKV was detected on Yap Island in the Federated States of Micronesia. Forty-nine confirmed and 59 probable ZIKV infections were identified during the investigation (5). Results of a serosurvey indicated that approximately 73% of residents of the island had been infected with the virus, potentially indicating the ability of ZIKV to spread easily. In 2015, ZIKV was identified in Brazil (6). By the end of the year, an estimated 440,000 to 1,300,000 cases of ZIKV infection were projected to occur in the country (7). Because of the observed increase in microcephaly and neurological

disorders, such as Guillain-Barré syndrome, the World Health Organization (WHO) declared a Public Health Emergency of International Concern (PHEIC) on February 1, 2016 (8). In 2016, Florida experienced a large increase of travel-associated ZIKV infections (9). Later that summer, local transmission was detected in Miami-Dade County, resulting in 285 cases of ZIKV infection (10). By the summer of 2017, the Pan American Health Organization (PAHO) had reported a total of 794,053 suspected, confirmed, and imported cases of the disease in the Americas (11). Congenital abnormalities associated with these infections were confirmed in 3,539 of these cases. By 2019, ZIKV transmission had been reported in almost every country in the Americas. Although the outbreak has since slowed, the transmission of ZIKV is ongoing and continues to impact countries. During 2019, 33,896 ZIKV cases were reported to PAHO, 6,640 of which were confirmed cases (12).

The outbreak of ZIKV across the globe has elevated a once obscure disease to the forefront of medicine and public health. In the last four years (2016 through 2019), 6,707 citations were indexed in PubMed containing “Zika” in the article title or abstract as compared to only 141 before 2016 (13). Despite the increased opportunity and availability of data, many questions are left unanswered.

Original estimates of the incubation period of ZIKV infection and viral clearance are rooted in a single systematic review performed in 2016 shortly following the PHEIC announcement by the WHO (14). Twenty publications, with case data from only 25 individuals, were included. Notably, this review focused only on blood specimens (whole blood, serum, and plasma), as the objective of the study was to estimate risk of transfusion-transmitted infections through blood donation. Since the PHEIC declaration,

many more case reports, case series, and observational studies have been published. These reports not only offer an expanded body of work from which to draw conclusions, but often utilize a wider breadth of clinical specimens to detect the virus, including whole blood, serum/plasma, urine, saliva, semen, and others. Recent case reports indicate that the time to clearance within whole blood specimens may significantly differ than clearance rates in serum or plasma. In a prospective observational study conducted by Joguet and colleagues, ZIKV RNA was detected in 3 of 15 patients at 120 days post-symptom onset (15). Froeschl and colleagues also report a case exhibiting detection of ZIKV RNA longer than 100 days (16). In a point-to-point comparison of whole blood and plasma specimens, 23 whole blood specimens were positive as compared to 9 corresponding plasma specimens (17). Despite prolonged detection observed in some specimen types, viral culture is not widely performed. This is partly due to the difficulty of the procedure and also because of the observed low viral load observed in these specimens (18). These low viral loads may indicate the detection of RNA by rRT-PCR does not reflect infectious virus.

An update to this systematic review to include the most recent reports and covering a greater variety of specimen types is needed to refresh the current understanding of the virus. Understanding the detection profiles of different specimen types can assist healthcare providers and epidemiologists in better detecting infections. Further, detection of the virus in different specimen types may indicate the potential for other methods of transmission, such as via person-to-person contact.

Several studies offer a glimpse into the course of viral load associated with ZIKV. Most reports and studies rely on very small sample sizes and unstandardized specimen

sampling (19-24). These reports provide limited evidence for the duration of positivity by rRT-PCR, but due to unstandardized testing methods, sporadic nature of specimen collection, and the limited sample sizes, they can only offer broad generalizations of the viremia and viruria caused by ZIKV. Differences in viral shedding amongst different groups have been observed. Prolonged viremia has been observed in case reports of pregnant women (25, 26), indicating a potential relationship between pregnancy status and ability to clear the virus. Increased viral loads in pregnant cases would provide further evidence for this potential relationship. Because YFV and DENV are closely related to ZIKV, those previously vaccinated or infected may exhibit the ability to clear the virus more rapidly.

Understanding the complete course of viral load is especially important for developing and refining laboratory methodologies for the detection of ZIKV in clinical specimens. This knowledge can also aid epidemiologists in interpreting laboratory values and performing public health interventions. A more complete picture of the course of viral load can also assist investigators in understanding the viral kinetics of ZIKV when antiviral interventions are used as well as understanding the potential implications of viremia on pregnancy outcomes.

Given the nondescript nature of symptoms, laboratory diagnosis is critical for the identification of ZIKV infections. Several diagnostic methods were used to identify and confirm ZIKV infection in patients (18, 27). Viral RNA was detected by rRT-PCR assays and generally considered confirmatory when positive. Urine has been observed to last longer following infection and at higher levels than serum (24, 28), potentially making urine an ideal specimen type. Antibody response to ZIKV was detected by virus-specific

IgM and plaque reduction neutralization test (PRNT) assays. Because the IgM assay for ZIKV can produce false-positive results due to antigenic similarities with other flaviviruses, positive or equivocal IgM results were considered presumptive positive until confirmed by ZIKV- and DENV-specific PRNT (29). The PRNT was generally considered the "gold standard" for differentiation and confirmation of arbovirus infections. However, it has been demonstrated that significant immunological cross-reactivity between ZIKV and DENV exists (30-33), creating a significant challenge for diagnostic laboratories. These issues with the cross-reactivity may be exacerbated when considering populations with an increased proportion of exposure to DENV or pregnant cases.

Effective use of laboratory assays is critical in the diagnosis of arboviral infections. The CDC publishes its recommended testing guidance and algorithms (34, 35), revising them periodically as needed. Testing for ZIKV is grouped by clinical presentation (symptomatic or asymptomatic) and pregnancy status, due to the increased risk presented to developing fetuses. Significant changes were made between 2017 and 2019, including changing the specimen collection window for rRT-PCR testing and including DENV testing due to the cross-reactivity between both viruses.

Study Purpose and Significance

This study examines the natural history of viral load in ZIKV and its role in clinical diagnosis and outbreak detection. This is first accomplished through systematic review and analysis of published reports of symptomatic ZIKV infections. Viral load is further examined using epidemiological and laboratory data collected by the Florida

DOH from 2016 to 2017 during the ZIKV outbreak. Finally, the utility of testing guidelines recommended by the Centers for Disease Control and Prevention (CDC) in an outbreak setting are evaluated using the data from the Florida DOH.

The results of this study will help clinical and public health practitioners interpret the results from current diagnostic assays for ZIKV and potentially guide the development of future assays and testing guidelines. Additionally, increased knowledge of the characteristics of such tests will allow practitioners to target and adapt current screening programs to the appropriate populations. These results will also inform and guide public health actions in future endeavors to control the virus.

Specific Aims and Hypotheses

Aim 1: Using a systematic review, estimate the incubation period, infectious period (based on viral isolation results), and time to viral clearance in individuals infected with ZIKV in whole blood, serum/plasma, urine, saliva, and semen.

Hypothesis 1a: Detectable viral RNA in whole blood decreases over time slowest compared to the other specimen types.

Hypothesis 1b: The ability to isolate the virus in culture is very small, averaging no more than two weeks post-symptom onset.

Aim 2: Using Florida DOH laboratory and surveillance data, estimate the progression of viremia/viruria and viral clearance in individuals infected with ZIKV in whole blood, serum, and urine.

Hypothesis 2a: Detectable viremia/viruria in non-pregnant individuals, demonstrated by the presence of ZIKV RNA, decreases over time slower in whole blood specimens than in urine or serum specimens.

Hypothesis 2b: Detectable viral RNA in all three specimen types decreases over time more slowly in pregnant women than in non-pregnant individuals.

Hypothesis 2c: Detectable viral RNA in all three specimen types decreases faster over time in individuals previously infected with another flavivirus or vaccinated with the yellow fever vaccine than in those with no previous infection or vaccine history.

Aim 3: Using Florida DOH laboratory and surveillance data, evaluate the use and timing of molecular and serological testing to detect and differentiate ZIKV infections in an outbreak setting.

Hypothesis 3a: In specimens collected within two weeks of symptom onset, reverse-transcriptase polymerase chain reaction (RT-PCR) will detect a greater number of cases than IgM.

Hypothesis 3b: Among RT-PCR positive cases, urine will detect the greatest number of cases as compared to serum and whole blood.

Hypothesis 3c: Positive or equivocal DENV IgM and IgG results are more likely to be seen in cases born in countries endemic with dengue fever than those born in the United States.

Hypothesis 3d: Inconclusive plaque reduction neutralization test (PRNT) results are more likely to be seen in cases born in countries endemic with dengue fever than those born in the United States.

Hypothesis 3e: Inconclusive PRNT results are more likely to be seen in pregnant cases than those non-pregnant cases.

Hypothesis 3f: A diagnostic algorithm including DENV IgM and IgG testing would decrease the number of specimens required to be referred for PRNT testing.

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Chapter 2

Literature Review

Identification

The ZIKV belongs to the *Flaviviridae* family and is a member of the *Flavivirus* genus. Members of the *Flavivirus* genus are single-stranded, positive-sense RNA viruses, characterized by a type I cap at the 5' end and one open reading frame (ORF) that encodes for three structural proteins and seven nonstructural proteins (1, 2). The structural proteins, the capsid, membrane (M protein), and envelope (E protein), are critical to the formation of the viral particle. These nonstructural proteins are integral in the replication of the virus within the host cell. Each virion is approximately 50 nm in diameter and spherical in shape. The surface of the virus is composed of E and M proteins arranged symmetrically as an icosahedron (3). Several other clinically important species of viruses, including YFV, DENV, WNV, and St. Louis encephalitis virus, share the same genus as ZIKV.

The ZIKV was first isolated from a sentinel rhesus monkey in the April of 1947 (4). Researchers placed platforms of rhesus monkeys in the Zika Forest in Uganda, taking their temperatures daily. When a monkey exhibited a fever, blood specimens were taken and injected into both mice and other Rhesus monkeys to demonstrate infectivity. The following year, the virus was again isolated from *Aedes africanus* mosquitoes from the same forest. These isolates were characterized serologically by using antiserum to other viruses, such as YFV, DENV, WNV, and others, to neutralize activity of the virus before infecting mice. Through these preliminary studies, it was determined that the virus was a

distinct, previously undiscovered virus, and was named for the forest where it was first discovered. ZIKV was later isolated from a pool of *Ae. aegypti* in Malaysia in 1966 (5) where it was thought to be circulating amongst wild monkeys.

Emergence and Spread as a Human Pathogen

The first human infections with ZIKV reported in literature were from Nigeria in 1952 (6), although these were later thought to be ascribed to the incorrect virus (5, 7). It is now thought that a laboratory-acquired infection in a researcher residing in Uganda in 1962 is the first reported infection with ZIKV in a human (8). Numerous serosurveys have been conducted in Africa, Asia, and the Pacific islands in an attempt to determine the extent of spread and prevalence of the virus (9, 10). These studies, however, tend to suffer from complications arising from the serological cross-reactivity between ZIKV and other closely related viruses, and differences in testing methodologies, making their findings difficult to interpret.

The first documented outbreak of ZIKV infections occurred in 2007 in Yap State in the Federated States of Micronesia, located in the western Pacific Ocean. Before this outbreak, only 14 human infections had been previously reported (2). Of the 158 patients tested, 49 were confirmed to be infected with the virus, and 59 were considered probable infections (11). A household serosurvey of residents indicated that 73% of island residents aged 3 years and older had been infected with the virus, demonstrating the ability of the virus to establish itself in a community. This outbreak also provided

valuable clinical and epidemiological information on the symptoms and clinical progression of the disease (11).

In October of 2013, the first cases of ZIKV infections were recorded in the French Polynesia, located in the southern Pacific Ocean; by the end of the year, almost 6,000 suspect cases had been identified through public health surveillance systems, indicating up to 19,000 infections could be present (12). Approximately 11% of the country's population is estimated to have sought treatment for ZIKV during the outbreak, which ended in April 2014 (13). Phylogenetic analysis of virus from this outbreak demonstrated the strain to be closely related to an isolate from the Yap State outbreak as well as an isolate identified in Cambodia in 2010 (11). A serosurvey conducted 18 months later indicated that 49% (95% CI 42% to 57%) of study participants exhibited IgG antibodies to ZIKV (14). By the beginning of 2014 and into 2015, outbreaks of ZIKV infections began to appear in neighboring islands in the south Pacific Ocean, including New Caledonia, the Cook Islands, Easter Island, Vanuatu, the Solomon Islands, and others (13, 15-18).

Molecular evidence suggests that ZIKV may have been introduced into Brazil between May and December of 2013 (19, 20). However, the first reports of cases of mild illness presenting with rash did not begin appearing until early 2015. Between February and April 2015, almost 7,000 such cases had been reported (18). In March of 2015, seven sera from the state of Bahia, Brazil tested positive for ZIKV by reverse-transcriptase PCR (RT-PCR); phylogenetic analysis indicated that these isolates were most closely related to an isolate from the French Polynesia in 2013 (21). During the same month, a cluster of cases in the state of Rio Grande do Norte, Brazil was tested and identified as the first

report of autochthonous transmission of ZIKV in the country (22). Unfortunately, ZIKV infections did not become a reportable condition until 2016, making it challenging to ascertain the true extent of the outbreak in Brazil in 2015. In September, the Brazilian Ministry of Health (MOH) estimated that the country would experience between 443,502 and 1,301,140 cases of ZIKV infections by the end of 2015 (23). By 2016, the outbreak in Brazil had expanded to most of the country's states (24). By the end of 2016, 130,701 confirmed and 84,618 probable cases had been reported to the MOH (25). A survey in the state of Bahia conducted between 2015 and 2016, indicated a seroprevalence of 63.3% (95% CI 59.4% to 66.8%) (26).

Cases of ZIKV infection began appearing in neighboring countries towards the end of 2015 (27). The first cases of ZIKV infections acquired in Colombia were reported in October of 2015 (28). Suriname, El Salvador, Guatemala, Paraguay, Venezuela and Mexico reported locally acquired ZIKV infections in November of 2015 (27). On February 1, 2016, the Director-General of the WHO declared a PHEIC (29). By the end of 2016, 48 countries in the Americas region had reported autochthonous transmission of ZIKV (30). The Pan American Health Organization (PAHO) reported 534,553 probable and 177,614 confirmed cases of ZIKV infection in the Americas in 2016. Multiple phylogenetic analyses indicate that the introduction of ZIKV in the Americas originated in Brazil (20, 31). Further, molecular clock analyses are fairly consistent in estimating that the introductions into a country typically occurred months before public health surveillance systems identified these infections, indicating sustained transmission was ongoing during this time.

Between January 2015 and February 2016, 116 cases of ZIKV infection were reported in the United States (32), but none of these cases were acquired within its borders. By the end of July 2016, 383 travel-associated cases had been detected in the state of Florida (33). On August 1, 2016, in response to the identification of four locally acquired cases of ZIKV infections, the CDC issued travel guidance through the Health Alert Network, recommending pregnant women avoid unnecessary travel to the Wynwood area in Miami-Dade County, Florida (34). On August 16, 2016, the Florida Department of Health (DOH) announced another area of local transmission in Miami Beach, and on October 13, 2016, local transmission in the Little River neighborhood. As with other introductions, genetic analysis of 39 genomes associated with the outbreak in Miami-Dade County indicated that the virus was introduced at least two months before the initial cases were detected in July (35). At the conclusion of 2016, 285 locally acquired and 1,122 travel-associated cases of ZIKV infection had been identified in Florida. In 2017, the total number of ZIKV infections reported in the United States dropped more than 10-fold from 5,168 in 2016 to 452; only seven of these cases in 2017 were determined to be acquired locally (36, 37).

Although the magnitude of the ZIKV infection outbreak has decreased since its peak in 2016, and the PHEIC was announced over less than a year from its declaration, ZIKV still remains a concern in the Americas. As of July 2019, the WHO reported that at least 87 countries or territories had evidence of local transmission of ZIKV (38). Between 2017 and 2019, 123,009 total cases (30,019 confirmed) were reported in the Americas to PAHO with the majority of these cases coming from Brazil (39). By 2019, only three

countries in the Americas (mainland Chile, Uruguay, and Canada) had not reported ZIKV transmission (38).

Transmission and Vectors

The primary mode of transmission of ZIKV is via the bite of infected mosquitoes (40). There are two main transmission cycles associated with ZIKV transmission: sylvatic and urban. The sylvatic cycle refers to transmission between non-human primates and zoophilic mosquito species. Once transmission is established within a human population, transmission is primarily via the urban cycle, between human hosts and anthrophilic mosquito species. The species thought to be responsible for most vector borne transmission is the *Ae. aegypti* mosquito (40). This species has been implicated both through field collections and experimental transmission studies (9, 40). Other *Aedes* species, such as *Ae. albopictus* and *Ae. hensilii*, have also demonstrated the ability to transmit the virus. The current geographical distribution of *Ae. aegypti* closely resembles the spread of the ZIKV infections during the 2016-2018 outbreak. Modeling of vector distribution, indicated that, out of the 188 countries or territories suitable for *Ae. aegypti*, 85 (45%) had reported autochthonous transmission of ZIKV (41).

There are three other primary mechanisms of transmission for ZIKV: maternal-fetal transmission, blood product transfusions, and sexual transmission. Maternal-fetal transmission occurs when the virus passes from an infected mother to her infant. Congenital infection can occur if the virus crosses through the placenta and infects the unborn child (42). In a prospective cohort study of 291 pregnancies in French Guiana during the ZIKV infection outbreak, infection of the mother resulted in maternal-fetal

transmission in 26% of cases; no differences were observed between those that resulted in congenital infections and those that did not (43). The ZIKV has also been detected and cultured from breast milk (44, 45), representing another possible route of maternal-fetal transmission.

Probable transfusion-transmitted infections have also been reported in the literature (46, 47), although it is often difficult to completely rule out vector-borne transmission in these cases. During the 2013 outbreak in the French Polynesia, nucleic acid testing of blood donors identified 42 asymptomatic infections in a four-month period (48). In February 2016, the Food and Drug Administration issued guidance on donor deferral and testing for areas with and without local transmission in the United States (49). Between April and June 2016, ZIKV RNA was identified in 68 blood donors in Puerto Rico using newly implemented screening procedures for the virus (50), demonstrating the potential for transmission through the blood supply.

The first case of sexual transmission of ZIKV was likely a case report from 2008 (51). In this case, a male researcher that had recently returned to the United States from Senegal fell ill with ZIKV disease shortly after his return. Five days following the onset of his symptoms, his wife, having not recently traveled, also developed similar symptoms. Although it is impossible to rule out isolated vector-borne transmission or transmission via direct contact, the couple reported having sexual intercourse prior to the onset of symptoms. Several other reports of putative male-to-female sexual transmission have been published (52-54), as well as female-to-male (55), and male-to-male (56) sexual transmission. The ZIKV has been found in both seminal fluids (57-59) and vaginal secretions (60, 61). Both the WHO and CDC recommend that males practice safe sex for

at least three months and females practice safe sex for at least two months following possible exposure to ZIKV; women that are pregnant should practice safe sex with their partner for the duration of their pregnancy following possible exposure to the virus (62, 63).

Clinical Presentation

The first report of symptoms associated with ZIKV disease was in a volunteer experimentally infected with the virus (64). The patient experienced a slight headache, a short period of mild fever, and malaise approximately 82 hours following inoculation. On the fifth day of illness, the patient's headache increased in severity, fever rose slightly, and he experienced nausea and vertigo. The illness resolved on the morning of the 7th day with no other sequelae. Infection with ZIKV was confirmed by culturing the virus from the volunteer and observation of an increase in antibodies to the virus. A cohort of 31 patients from the Yap Island outbreak in 2007 provide the first sizeable sample of people with ZIKV infection symptoms (11). The most common symptom experienced by the cohort was a maculopapular or papular rash (90% of patients), followed by fever (65%), arthritis or arthralgia (65%), nonpurulent conjunctivitis (55%), myalgia (48%), headache (45%), retro-orbital pain (39%), edema (19%), and vomiting (10%). Rash duration was approximately 6 days (with a range of 2 to 14 days), and arthralgia lasted for approximately 3.5 days (1 to 14 days). Symptoms were generally mild with no hospitalizations or deaths. A systematic review of 52 additional articles and reports conducted in 2016 found that the most frequently reported symptoms were the same as those described in the Yap Island outbreak (65).

Another key finding of the Yap Island outbreak was the ratio of symptomatic illness to those presenting without symptoms. The authors estimated that approximately 919 residents (18%) infected with ZIKV presented with clinical symptoms (11). This indicates that approximately 80% of ZIKV infections present with no symptoms. A systematic review conducted in 2018 identified 23 studies describing the prevalence of asymptomatic infections (66). The authors observed a large amount of variation in the prevalence of asymptomatic infections, ranging from 29% to 82%, depending on the study population. The pooled prevalence of asymptomatic infections was 61.8% (95% CI 33.0% to 87.1%), but the authors note that this estimate may not be robust. Other arboviruses demonstrate varying prevalence of asymptomatic infections. Approximately 75 to 80% of infections with WNV and DENV are thought to be asymptomatic (67, 68);whereas only about 55% of infections with YFV are thought to be asymptomatic (69). However, because most infections with ZIKV that do present with symptoms are mild in nature (9, 11), these estimates could be too high.

With the introduction and dramatic transmission of ZIKV in Brazil, a marked increase in the number of cases of microcephaly, a birth defect where a newborn's head circumference is smaller than normal, was also observed (70). Between 2000 and 2014, the average annual number of reported cases of microcephaly in Brazil was 157.3; in 2015, 574 cases of microcephaly were reported. A similar increase in microcephaly was observed in Columbia in 2016; 476 cases of microcephaly were reported between epidemiologic weeks 5 and 45 as compared to 110 cases during the same time period the year prior (71). Retrospective analysis of cases during the 2013–2015 French Polynesia outbreak indicated a similar trend (72). This correlation, in part, led the WHO to declare

the PHEIC in February 2016 (29). A pattern of other birth defects and anomalies emerged beyond microcephaly. In addition to severe microcephaly, characteristics of congenital Zika syndrome (CZS) include brain anomalies, ocular anomalies, congenital contractures, and neurological complications (73). In a study of 1,450 children aged ≥1 year born to mothers with ZIKV infection in the United States or its territories, 203 (14%) had at least one ZIKV-related birth defect or neurodevelopmental abnormality (74).

A similar correlation was seen with ZIKV and Guillain-Barré syndrome (GBS), a disorder where the patient's immune system damages the nervous system, causing weakness or paralysis. During the ZIKV disease outbreak in the French Polynesia, the incidence rate of GBS was approximately 20 times higher than expected (13). A case-control study of 42 French Polynesian patients diagnosed with GBS, 41 (98%) were positive for IgM or IgG against ZIKV compared with only 35 (36%) of the controls (OR 59.7, 95% CI 10.4 to ∞) (75). ZIKV was detected in two patients who were diagnosed with GBS in Martinique (76). Out of 56 patients diagnosed with GBS in Puerto Rico during the first seven months of 2016, 34 (37%) had evidence of ZIKV or flavivirus infection (77). As of October 2016, the WHO reported that 13 countries had observed an increase in the incidence of GBS cases; an additional six countries had reported GBS in patients diagnosed with ZIKV infection (78). A systematic review conducted in 2018 indicated that the incidence rate for Latin American and Caribbean nations increased 2.6 times (95% CI 2.3 to 2.9) during the ZIKV disease outbreak (79).

Laboratory Methods

Three types of diagnostic assays are predominantly used to identify infections with ZIKV (80, 81). The first group are those tests that detect the virus' RNA, or nucleic acid amplification tests (NAATs). Although there are many variations of these tests, NAATs detect a genetic sequence (or multiple sequences) of viral RNA specific to ZIKV and amplify the sequence for detection. These assays are both sensitive and specific, allowing for the identification of very minute quantities of RNA. Because these assays detect the virus directly, a positive result in a patient typically indicates an acute infection with the virus (81, 82).The most common type of NAAT is the real-time reverse-transcriptase polymerase chain reaction (rRT-PCR) test. Briefly, these assays mimic the natural process of DNA replication by first producing complementary DNA (cDNA) from the virus' RNA, binding ZIKV-specific primers to the cDNA, and replicating using a thermostable polymerase (83). Detection is facilitated in "real-time" with fluorescently labeled probes or other detection chemistries. Reactions can also be multiplexed, meaning multiple targets (e.g., multiple viruses) can be detected simultaneously (84). Common genetic targets of current ZIKV NAATs include the envelope, membrane, and several of the nonstructural proteins (81, 85). Even though rRT-PCR assays are able to detect very small quantities of virus, the viral load associated with ZIKV infections has been reported to be substantially lower than other flavivirus infections (86, 87) and may differ between specimen types (88).

Another common assay type for the diagnosis of ZIKV infection is the enzyme-linked immunosorbent assay (ELISA). These assays most frequently look for the presence of IgM or IgG antibodies against the virus produced by the patient. IgM

antibodies are typically produced in response to the first time the immune system encounters a new pathogen and are typically present shortly after infection until a few months. As the quantity of IgM antibodies wanes, the amount of IgG antibodies, which typically provide long lasting immunity, increases and often lasts for years. Generally, the presence of IgM antibodies indicates that the infection was recent (within the last few weeks or months); whereas the presence of IgG indicates a previous infection (82). Of the five assays currently approved for use in the United States, all of them detect only IgM (89, 90); ZIKV IgG assays exist but are not widely used. IgM ELISAs work by coating a test well with anti-IgM antibodies that will capture the patient's IgM antibodies from serum. These bound antibodies are then exposed to a known ZIKV antigen. This process is detected by adding a fluorescently labeled antibody that will bind to the antigen and produce a color change. The antigen used for these assays vary, but usually consist of a non-infectious ZIKV-like particle, the ZIKV envelope, or the ZIKV NS1 protein (85). The presence of IgM antibodies would indicate that the patient has previously been infected by the virus, although the timing of such infection is often difficult to intuit. A recent study demonstrated that 76% of patients with symptomatic ZIKV infection (confirmed by rRT-PCR) had detectable IgM against ZIKV 25 months following the initial illness (91), limiting the utility of this test as an indicator of recent infection. Another complication of these assays is the cross-reactivity observed with other flaviviruses, especially DENV (81, 85, 92, 93). Under current diagnostic guidance from the CDC, specimens that test positive for ZIKV IgM should be tested further (82).

The plaque reduction neutralization test (PRNT) is considered the "gold standard" for serological testing of ZIKV (85). This assay determines if patient antibodies are

effective at neutralizing live virus to keep it from replicating. The assay can be conducted with ZIKV, DENV, and other flaviviruses. Patient sera is serially diluted and incubated with a standard amount of virus (81). This mixture is then inoculated onto a monolayer of cultured cells on a semisolid medium and incubated. Viral plaques are then counted, and the dilution resulting in a reduction in 90% of the plaques is considered the endpoint of the assay. This process is both time- and labor-intensive and typically only available in select public health laboratories. Because of the cross-reactivity observed in the ZIKV IgM ELISA, PRNT for both ZIKV and DENV is recommended for positive or equivocal results (82). However, recent experiences with the PRNT assay have indicated that the assay is also prone to cross-reactivity with DENV (11, 93), causing difficulty in interpretation in populations previously exposed to DENV and limiting its utility.

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Chapter 3

Persistence of Zika Virus in Clinical Specimens: A Systematic Review

Background

First isolated in 1947 (1), only 14 cases of human ZIKV infection had been reported between the discovery of the virus and 2007 (2). However, with the recent global spread of the virus, a dramatic increase in the number of confirmed and presumptive cases has been reported (3-6). Despite this impressive surge in cases, little is known about the time course of viremia and viruria. As the literature expands to include case reports and observational studies, it is important to coalesce these data to have an accurate and updated understanding of viral persistence in those infected with the virus.

Understanding the natural history of viral persistence is especially important for healthcare providers and epidemiologists in interpreting laboratory values and performing public health interventions. This knowledge can also help in developing laboratory methodologies for the detection of the virus and refining testing algorithms. A more complete picture of when ZIKV RNA can be detected in various clinical specimens can also assist researchers in understanding the kinetics of the virus when antiviral interventions are used as well as interpreting the potential implications of viral load on pregnancy outcomes. To this end, a systematic search and review of available literature was performed to identify published articles and other materials describing the progression of the virus in various body fluids in uncomplicated, symptomatic ZIKV infections.

Methods

Protocol. This systematic review was conducted according to the 2009 Preferred Reporting Items for Systematic Reviews and Meta-Analyses Guidelines (7) and the registered protocol (CRD42018092049) can be found on PROSPERO (8).

Eligibility. Original case reports of symptomatic ZIKV infection in humans were included. Cases without symptom onset or those where infection was putatively acquired *in utero* were excluded as the timing of viremia could not be reliably determined. Cases presented along with significant medical comorbidities (such as simultaneous co-infections, chronic health conditions, or compromised immune status) or as part of an intervention were excluded in an attempt to limit confounding. Case data from the validation of laboratory methods were also excluded as to not potentially count cases more than once.

Outcomes. Two primary outcome measures were examined: exposure and viral clearance intervals. The exposure interval was defined as the first to the last potential date of exposure, as determined by the authors presenting the case(s). Where possible, the viral clearance interval was defined as the date of last positive RT-PCR result to the first negative RT-PCR result. When no negative result was reported, the last positive RT-PCR result was utilized as the start of the viral clearance interval and left unbounded during statistical analysis. Intervals were calculated on whole days only.

Search methodology. A literature search of the CINAHL, EMBASE, LILACS, and Medline electronic databases was conducted independently by two authors (SLW and MC) on March 22, 2018. The following generic search phrase was used in each database, modified to include MeSH terms and subject headings where appropriate: Zika OR ZIKV

AND viremia OR viraemia OR viruria OR kinetic* OR dynamic* OR progress* OR "time course" OR clearance OR ((viral OR virus) AND (load OR isolation OR culture)) OR PCR. No limitations were included in the search; all records included up to the date of the search were included. Covidence Systematic Review Software (Veritas Health Innovation, Melbourne, Australia) was used to import and screen references. Reviews were completed independently by two of the authors (SLW and MC). Titles and abstracts were evaluated for preliminary inclusion in the study by both reviewers. If either reviewer selected a study for inclusion, the full text was evaluated to ensure applicability to the review.

Table 1. Database search phrases

Database	Search Phrase
CINAHL Current Nursing and Allied Health Literature	(Zika OR ZIKV OR (MH "Zika Virus Infections") OR (MH "Zika Virus")) AND (viremia OR viraemia OR viruria OR kinetic* OR dynamic* OR progress* OR "time course" OR clearance OR ((viral OR virus) AND (load OR isolation OR culture)) OR PCR OR (MH "Viremia") OR (MH "Kinetics") OR (MH "Disease Progression") OR (MH "Viral Load") OR (MH "Microbial Culture and Sensitivity Tests") OR (MH "Polymerase Chain Reaction"))
EMBASE Excerpta Medica dataBASE	(Zika OR ZIKV OR 'Zika fever'/de OR 'Zika virus'/de) AND (viremia OR viraemia OR viruria OR kinetic* OR dynamic* OR progress* OR "time course" OR clearance OR ((viral OR virus) AND (load OR isolation OR culture)) OR PCR OR 'viremia'/de OR 'viruria'/de OR 'kinetics'/de OR 'viral clearance'/de OR 'disease exacerbation'/de OR 'virus load'/de OR 'virus isolation'/de OR 'virus culture'/de OR 'pcr'/de OR 'polymerase chain reaction'/exp)
LILACS Literatura Latino-Americana e do Caribe em Ciências de Saúde	(Zika OR ZIKV) AND (viremia OR viraemia OR viruria OR kinetic* OR dynamic* OR progress* OR "time course" OR clearance OR ((viral OR virus) AND (load OR isolation OR culture)) OR PCR)
MEDLINE Medical Literature Analysis and Retrieval System Online	(Zika OR ZIKV OR MESH.EXACT("Zika Virus Infection") OR MESH.EXACT("Zika Virus")) AND (viremia OR viraemia OR MESH.EXACT("Viremia") OR kinetic* OR MESH.EXACT("Kinetics") OR dynamic* OR progress* OR MESH.EXACT("Disease Progression") OR "time course" OR ((viral OR virus) AND (load OR isolation OR culture)) OR MESH.EXACT("Viral Load") OR MESH.EXACT("Virus Cultivation") OR PCR OR MESH.EXACT.EXPLODE("Polymerase Chain Reaction"))

Data collection. Each record was assigned a reference number and each case presented within that record was given a unique subject ID number. For each case, the following information was extracted when presented: gender, age, special population

characteristics (e.g. pregnancy status, flavivirus vaccinations, neurological involvement), number of symptoms, putative country of infection, specimen type, first and last day of potential exposure, first and last day of symptom onset, date of symptom resolution, specimen type, RT-PCR test results, cell line, timing of specimen collection, incubation timing, and detection methodology. Specimen types were simplified into the following categories: blood, cerebrospinal fluid (CSF), saliva, semen, serum, urine, vaginal, unknown, and other. Testing data were separated by specimen type. In other words, each line in the complete dataset represents a single patient and a single specimen type. For example, if one patient had three specimen types collected and tested, there would be three separate entries for that patient. Data were extracted by a single author (SLW) and reviewed for accuracy by a second author (MC); discrepancies were resolved by consensus.

Quality assessment. To evaluate the quality of each record and the risk of bias, the National Institute of Health's Study Quality Assessment Tools for case series studies was adapted. This tool evaluates studies on elements of the study population, description of the cases, uniformity of results, methods, and presentation (Figure 1).

Figure 1. Record quality assessment criteria.

	Criteria	Maximum Score
1	Does the patient(s) represent(s) the whole experience of the investigator (center)? 1 point for a single case report 1 point for poorly described case series 2 points for case series with described selection method	2
2	Was the exposure adequately ascertained? 1 point for well-described case history	1
3	Was the outcome adequately ascertained? 0 points for poorly described primary outcomes or if outcomes are unclear 1 point if primary outcomes are not systematically described amongst cases 2 points if primary outcomes are thoroughly and systematically described amongst all cases	2
4	Were other alternative causes that may explain the observation ruled out? 0 points if medical history not provided 1 point if medical history is provided or if additional lab testing provided	1
5	Was follow-up long enough for outcomes to occur? 0 points if testing is only conducted at one time point per specimen 2 points if serial testing performed	2
6	Is the case(s) described with sufficient details to allow other investigators to replicate the research or allow practitioners to make inferences related to their own practice? 0 points if case details are not provided 1 point if case details are unclear or incomplete 2 points if details are systematically collected and complete for all cases	2
7	Was a more advanced study design, such as a cohort design, utilized? 0 points if only a case study or case series 2 points if cohort study conducted	2

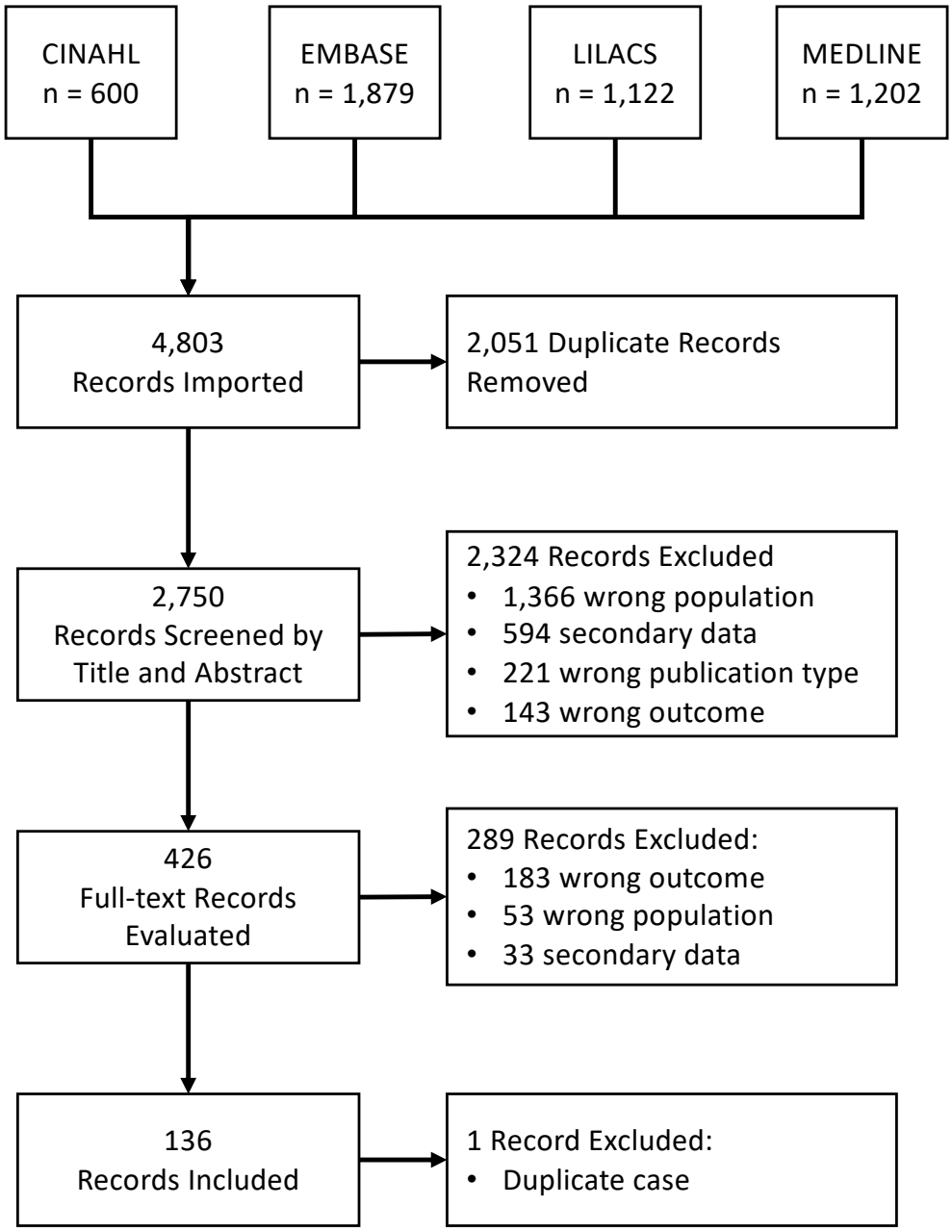
Statistical analysis. Descriptive statistics were calculated using SAS software version 9.4 (Cary, NC, USA). Incubation period and viral clearance rates were estimated using methods similar to those described by Lessler and colleagues (9). Briefly, upper and lower bounds were extracted from case data for the time of exposure, time of symptom onset, first and last positive RT-PCR results, and first negative RT-PCR result. Using Markov chain Monte Carlo (MCMC) modeling, these bounds were used to estimate the incubation period of ZIKV disease (time from exposure to symptom onset) and the time to viral clearance (from symptom onset). MCMC modeling was conducted using R statistical language (Vienna, Austria) with the JAGS package, version 4.10.

Results

Search Results

4,803 references (Figure 2) were identified from CINAHL (n = 600), EMBASE (n = 1,879), LILACS (n = 1,122), and MEDLINE (n = 1,202). Of these, 2,051 were identified as duplicate references and removed. The titles and abstracts of the remaining 2,750 references were screened for inclusion. 2,324 were excluded, leaving 426 full-text articles to be evaluated. Of these 426 articles, 289 were excluded, leaving 137 citations for inclusion into the study. One of these citations was a follow-up report on a previously included article.

Figure 2. Record selection flow chart.



Record Quality

Of the 137 records included in the study, 136 were reviewed for quality; the remaining record was an update to another study already included. The possible range of

scores was from 0 to 12, higher scores indicating a higher quality. The median score was 7 with a range of 0 to 12 (Table 2).

Table 2. Frequency of record quality scores.

Possible Score	No. Records
0	2
1	0
2	0
3	1
4	11
5	17
6	21
7	26
8	28
9	15
10	7
11	6
12	2

Patient Results

Data were abstracted from 792 patients from the 136 records. A median of 2 specimen types were reported for each patient (range: 1 to 6), resulting in 1,571 total entries. Availability of data varied greatly amongst the records. Amongst the patient-level data, gender was missing from 180 patients (22.7%), and age was available for only 65.2% of patients (Table 3). Complete exposure data was available for only 93 (11.7%) cases. Data on symptom presentation were available on less than half of all cases. Notably, the putative source of infection was presented in the majority of cases (77.8%). Specimen type was available in 99.2% (1,559) of specimen-level data. Unsurprisingly,

the availability of testing data varied. The earliest positive result was available in 67.9% of specimens (1,067), but the last positive result was presented in only 15.7% of records. A negative result, however, was available in about half of the specimens (53.2%).

Table 3. Availability of patient- and specimen-level data available.

	Female n (%)	Male n (%)	Unknown n (%)	Total n (%)
Total	285	327	180	792
Age*	256 (89.8)	260 (79.5)	0 (0.0)	516 (65.2)
Symptoms*	122 (42.8)	147 (45.0)	50 (27.8)	319 (40.3)
Source of infection*	255 (89.5)	283 (86.5)	78 (43.3)	616 (77.8)
Exposure period*	48 (16.8)	75 (22.9)	0 (0.0)	123 (15.5)
Symptom duration*	35 (12.3)	53 (18.6)	0 (0.0)	88 (11.1)
Exposure period and symptom onset*	41 (14.4)	52 (14.1)	0 (0.0)	93 (11.7)
Specimen type	526 (99.6)	684 (98.6)	349 (100.0)	1559 (99.2)
Earliest positive result	357 (67.6)	444 (84.1)	266 (50.4)	1067 (67.9)
Last positive result	75 (14.2)	149 (28.2)	23 (4.4)	247 (15.7)
First negative result	279 (52.8)	430 (81.4)	126 (23.9)	835 (53.2)
All three	53 (10.0)	114 (16.4)	20 (5.7)	187 (11.9)
PCR assay type	495 (93.8)	638 (91.9)	328 (94.0)	1461 (93.0)

*Age, symptoms, source of infection, exposure period, and symptom duration are derived from patient-level data (n = 792); all others derived from specimen-level data (n = 1,571).

285 (36.0%) of the patients were female and 327 (41.3%) were male; the gender of 180 (22.7%) patients was not described (Table 4). Thirty-six (12.3%) of the female

patients were pregnant. Thirty-five (4.4%) of the cases exhibited neurological complications, such as GBS or encephalitis.

Table 4. Case characteristics.

	Female n (%)	Male n (%)	Unknown n (%)	Total n (%)
Total	285 (36.0)	327 (41.3)	180 (22.7)	792
Age (years)				
Mean	32.8	34.7	-	33.7
Range	0 to 80	1 to 80	-	0 to 80
0-4	10 (3.5)	5 (1.5)	0 (0.0)	15 (1.9)
5-9	16 (5.6)	18 (5.5)	0 (0.0)	34 (4.3)
10-19	31 (10.9)	28 (8.6)	0 (0.0)	59 (7.5)
20-29	53 (18.6)	41 (12.5)	0 (0.0)	94 (11.9)
30-39	58 (20.4)	62 (19.0)	0 (0.0)	120 (15.2)
40-49	42 (14.7)	66 (20.2)	0 (0.0)	108 (13.6)
50-59	27 (9.5)	22 (6.7)	0 (0.0)	49 (6.2)
≥60	19 (6.7)	18 (5.5)	0 (0.0)	37 (4.7)
Not described	29 (10.2)	67 (20.5)	180 (100.0)	276 (34.9)
Special population				
Pregnant	36 (12.3)	-	-	36 (4.6)
Neurological complications	14 (4.9)	21 (6.4)	0 (0.0)	35 (4.4)
Number of key symptoms				
Zero	2 (0.7)	1 (0.3)	2 (1.1)	5 (0.6)
One	18 (6.3)	19 (5.8)	7 (3.9)	44 (5.6)
Two	30 (10.5)	41 (12.5)	15 (8.3)	86 (10.9)
Three	26 (9.1)	37 (11.3)	16 (8.9)	79 (10.0)
Four	34 (11.9)	35 (10.7)	9 (5.0)	78 (9.8)
Five	12 (4.2)	12 (3.7)	0 (0.0)	24 (3.0)
Six	0 (0.0)	2 (0.6)	1 (0.6)	3 (0.4)
Not described	163 (57.2)	180 (55.0)	130 (72.2)	473 (59.7)

Serum specimens were collected most often, representing approximately half of the dataset (46.0%); urine specimens represented 27.1% of the dataset (Table 5). Notably,

semen specimens represent 14.6% (101) of the specimens tested for male patients; whereas vaginal specimens only accounted for 5.3% (28) of those collected on female patients. Interestingly, given the heightened attention on neurological involvement, CSF specimens only represented 1.7% (27) of the dataset.

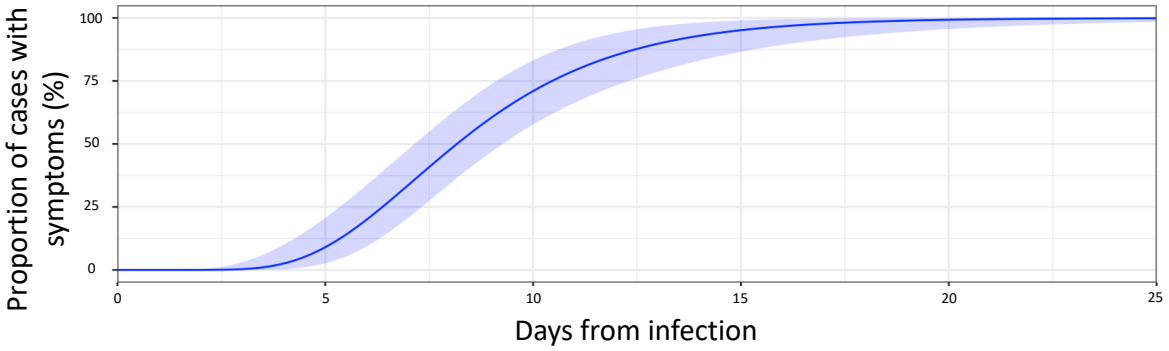
Table 5. Specimen types by gender.

Specimen type	Female n (%) 528	Male n (%) 694	Unknown n (%) 349	Total n (%) 1,571
Blood	31 (5.9)	35 (5.0)	15 (4.3)	81 (5.6)
Cerebrospinal fluid	10 (1.9)	17 (2.5)	0 (0.0%)	27 (1.7)
Saliva	35 (6.6)	56 (8.1)	56 (16.1)	147 (9.4)
Semen	-	101 (14.6)	0 (0.0%)	101 (6.4)
Serum	257 (48.67)	287 (41.4)	179 (51.3)	723 (46.0)
Urine	150 (28.4)	177 (25.5)	99 (28.4)	426 (27.1)
Vaginal	28 (5.3)	-	0 (0.0%)	28 (1.8)
Other	15 (2.84)	11 (1.6)	0 (0.0%)	26 (1.7)
Unknown	2 (0.4)	10 (1.4)	0 (0.0%)	12 (0.8)

The exposure period and date of symptom onset (necessary to estimate the incubation period) was only abstracted from 93 (11.7%) cases. The median exposure period was 14 days with a range of 0 to 547 days (Table 6). The median time from the last exposure to symptom onset was 2 days with a range of -10 to 59 days. Using MCMC modeling, the median incubation period was determined to be 6.5 days (95% credible interval [CrI]: 5.5 to 7.7 days) with a dispersion 1.7 days (95% CrI: 1.5 to 2.7 days) (Figure 3). The median duration of symptoms was 7 days with a range of 1 to 60 days.

No significant differences were observed between genders. The median time to earliest positive result post-symptom onset for all specimen types was 4 days with a range of -2 to 98 days. The median time to latest positive and first negative result for all specimen types was 15.5 (range 2 to 188) and 11.0 (range 0 to 256), respectively. Significant differences were observed between males and females for the timing of the first positive result and first negative result, but not the latest positive result.

Figure 3. Estimated incubation period for symptomatic Zika virus disease.



Solid line indicates estimated proportion of cases developing symptoms at days post-infection. Shaded areas indicate 95% CrI.

Table 6. Primary outcomes by gender.

Variable	n (%)	Median	Interquartile Range	Range	p-value
Exposure period					0.467
Female	48 (39.0)	14.5	16.5	2 to 547	
Male	75 (60.1)	12.0	19.0	0 to 365	
Total	123 (100.0)	14.0	17.0	0 to 547	
Symptom onset					0.883
Female	96 (45.7)	2.0	4.0	-8 to 6	
Male	114 (54.3)	1.0	5.0	-10 to 59	
Total	210 (100.0)	2.0	5.0	-10 to 59	
Symptom duration					0.185
Female	35 (39.8)	8.0	8.0	2 to 60	
Male	53 (60.2)	7.0	6.0	1 to 33	
Total	88 (100.0)	7.0	6.5	1 to 60	
DCPSO* earliest positive					<0.001
Female	357 (44.6)	3.0	3.0	-2 to 98	
Male	444 (55.4)	5.0	4.0	-2 to 91	
Total	801 (100.0)	4.0	5.0	-2 to 98	
DCPSO* latest positive					0.134
Female	75 (33.5)	14.0	24.0	2 to 120	
Male	149 (66.5)	18.0	36.0	2 to 188	
Total	224 (100.0)	15.5	34.5	2 to 188	
DCPSO* first negative					0.006
Female	279 (39.4)	9.0	17.0	0 to 198	
Male	430 (60.6)	13.0	25.0	0 to 256	
Total	709 (100.0)	11.0	20.0	0 to 256	

*days collected post-symptom onset

Differences were also observed when examining the timing of results for different specimen types (Table 7). For earliest positive results, blood, saliva, serum, urine, and vaginal specimens all had a similar median time to first detection (3 to 5 days). CSF

appeared to have a slightly longer time to become positive (median 8 days), although this was observed within a fairly small sample size. Semen specimens, however, did not become positive until a median of 15 days. There also appeared to be variation amongst specimen types for the first negative result. Serum specimens were negative within a median of 6 days post-symptom onset (range 0 to 256); whereas urine specimens were negative within a median of 20 days (range 0 to 134) and semen 42 days (range 4 to 201).

Similarly, viral clearance estimates varied significantly by specimen type (Table 8, Figure 4). Blood specimens had the longest mean time to viral clearance, 49.2 days (95% CrI: 33.9 to 80.8 days). Vaginal specimens had the shortest mean time to viral clearance (9.9 days, 95% CrI: 6.6 to 17.9 days). The time to viral clearance for urine was determined to be almost twice as long as that of serum (20.5 versus 10.8 days).

Table 7. Median times to first and last positive and first negative rRT-PCR results.

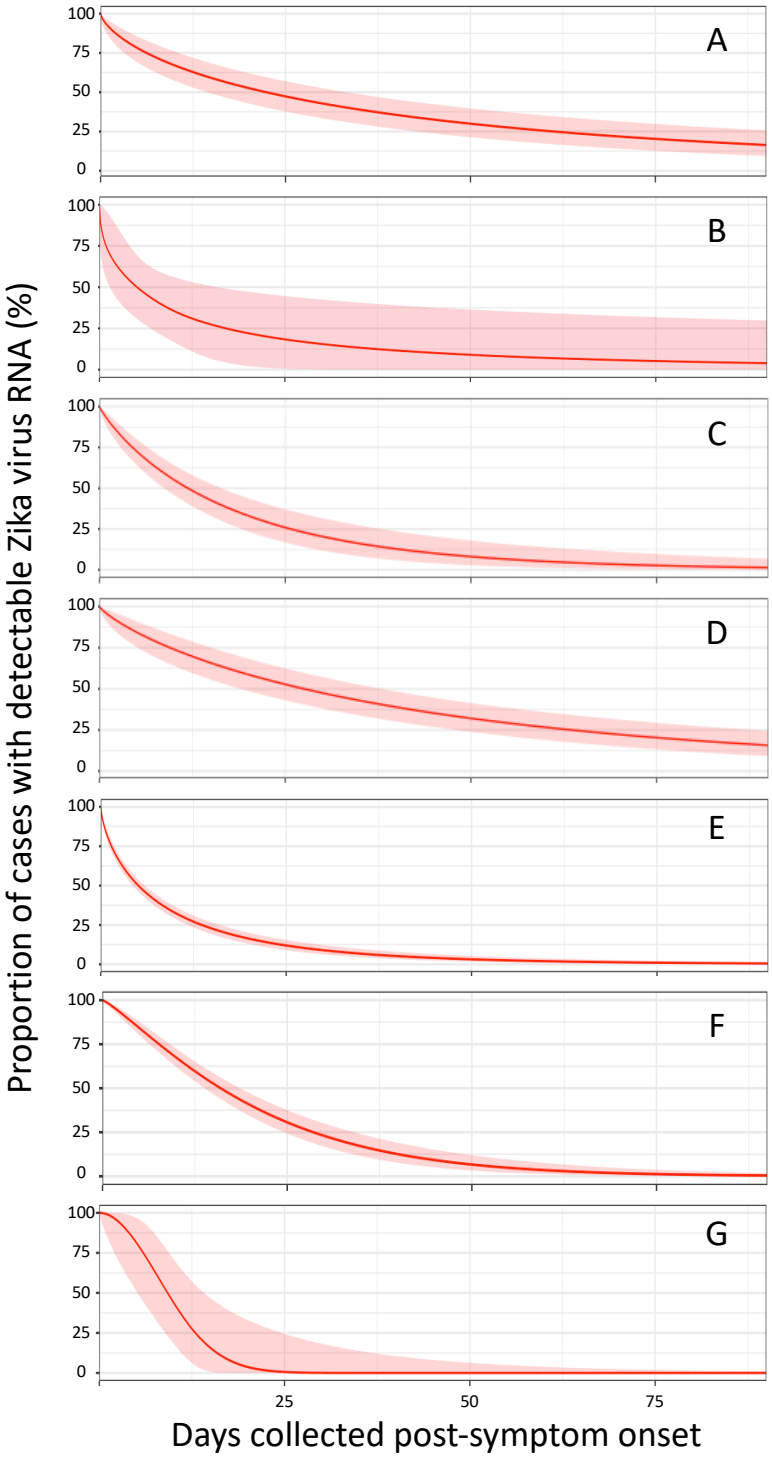
	Blood	CSF	Saliva	Semen	Serum	Urine	Vaginal	Other	Unknown	p-value
DCPSO Earliest Positive										
n	69	10	108	65	432	348	21	31	2	< 0.001
Median	5	8	4	15	3	4	4	3	6	
IQR	5	3	3	20	3	5	5	2	8	
Range	0 to 98	6 to 16	-2 to 39	3 to 91	-2 to 46	-1 to 91	0 to 37	1 to 8	2 to 10	
DCPSO Latest Positive										
n	39	0	25	37	53	84	5	12	1	< 0.001
Median	8	-	13	50	11	13.5	12	7	12	
IQR	83	-	22	50	24	10.5	1	2.5	0	
Range	1 to 140	-	3 to 91	11 to 188	1 to 120	2 to 80	11 to 14	2 to 18	12	
DCPSO First Negative										
n	61	18	68	82	404	162	25	27	0	< 0.001
Median	20	14	13	42	6	20	15	5	-	
IQR	84	9	15.5	67	9	21	13	5	-	
Range	1 to 140	4 to 27	0 to 134	4 to 201	0 to 256	0 to 134	7 to 197	0 to 198	-	

CSF: cerebrospinal fluid; IQR: interquartile ratio
* Kruskal-Wallis Test

Table 8. Time in days from symptom onset to viral clearance by specimen type.

	Blood	CSF	Saliva	Semen	Serum	Urine	Vaginal
n	169	28	186	159	889	559	45
Median	49.2	19.5	18.9	47.7	10.8	20.5	9.9
95% CrI	33.9 to 80.8	5.9 to 663.6	13.9 to 28.9	35.9 to 69.4	9.2 to 13.1	17.6 to 24.3	6.6 to 17.9

Figure 4. Estimated percentage of patients with virus detectable by rRT-PCR by specimen type.



A: blood, B: CSF, C: saliva, D: semen, E: serum, F: urine, G: vaginal. Shaded areas indicate 95% CrI.

Viral isolation data were available for 119 cases and 417 individual specimens. Serum was the specimen type most commonly cultured, followed by both semen and urine (Table 9). Overall, only 38 (13.9%) viral isolation attempts were successful. Semen, serum, and urine all exhibited similar recovery rates (15.8% to 19.8%). Notably, the recovery rate of blood and saliva was quite low. Vero (African monkey kidney) cell lines were utilized most often, followed by C6/36 (*Ae. albopictus*) cell lines; both had similar isolation recovery rates (14.9% and 16.7%, respectively). The timing of specimen collection for isolation attempts ranged from 0 to 201 days post-symptom onset. Interestingly, there was no significant difference observed in the ability to successfully culture ZIKV over time; recovery rates are fairly similar through first four weeks following symptom onset but decreases thereafter.

Table 9. Viral culture attempts.

	Successful n (%)	Unsuccessful n (%)	Total n (%)	
Total	58 (13.9)	359 (86.1)	417 (100.0)	p-value
Specimen type				0.033
Blood	0 (0.0)	33 (100.0)	33 (7.9)	
CSF	0 (0.0)	2 (100.0)	2 (0.5)	
Saliva	3 (5.4)	53 (94.6)	56 (13.4)	
Semen	18 (19.8)	73 (80.2)	91 (21.8)	
Serum	19 (15.8)	101 (84.2)	120 (28.8)	
Urine	16 (17.6)	75 (82.4)	91 (21.8)	
Other	2 (8.3)	22 (91.7)	24 (5.8)	
Cell line				0.017
BALB/c	3 (10.3)	26 (89.7)	29 (7.0)	
C6/36	17 (16.7)	85 (83.3)	102 (24.5)	
LLC-MK2	0 (0.0)	17 (100.0)	17 (4.1)	
Vero*	35 (14.9)	200 (85.1)	235 (56.4)	
Unknown	1 (3.2)	30 (96.8)	31 (7.4)	
Other	2 (66.7)	1 (33.3)	3 (0.7)	
DCPSO†				0.221
≤7	23 (16.3)	118 (83.7)	141 (33.8)	
8-14	7 (20.6)	27 (79.4)	34 (8.2)	
15-21	6 (15.8)	32 (84.2)	38 (9.1)	
22-28	4 (19.0)	17 (81.0)	21 (5.0)	
≥29	7 (6.7)	97 (93.3)	104 (24.9)	
Unknown	11 (13.9)	68 (86.1)	79 (18.9)	

*Vero lines included Vero, Vero-E6, and Vero-B4

† Days collected post symptom onset

Discussion

The incubation period of ZIKV infection in symptomatic patients was estimated to be 6.5 days. This is very similar to the estimate of 5.9 days by Lessler and colleagues (9). This estimate is also similar to a study published in 2017 that found the median incubation period to be 5.8 days (10) and another that calculated the median incubation period to be 6.8 days (11).

In the Lessler study, the time to viral clearance in serum specimens was found to be 9.9 days. The current study found the time to viral clearance in serum to be 10.8 days. The current study adapted Lessler's methods and increased the sample size through conducting an updated systematic review following the ZIKV pandemic. It should also be noted that Lessler's analysis evaluated viral clearance from infection as opposed to date of symptom onset, as in the current study. These results differ slightly with viral clearance estimates calculated in a prospective cohort followed for six months (12). In that cohort, serum had a median time to viral clearance of 15 days, and the median clearance time in urine specimens was 11 days. This is nearly half as long as the estimate of 20.5 days derived from this systematic review. The estimates for median viral clearance in semen, however, were similar (42 days in the prospective cohort versus 47.7 days in this study). The present study's results for blood, serum, and urine also closely matched those of another prospective cohort conducted after this systematic review (13). In that cohort study, whole blood specimens were observed to be positive in 89% of specimens collected through 79 days post-symptom onset. Urine positivity dropped from 75% in specimens collected between 12 and 19 DCPSO to 14% in those collected between 20 and 36 DCPSO. Although all specimens in that cohort were collected starting at day six post-symptom onset, none of the 116 serum specimens were positive, indicating rapid viral clearance from serum.

The availability and quality of viral isolation data varied significantly amongst records making analysis challenging. The substantial variability of isolation methodologies, from chosen cell line to number of passages and detection methods, makes comparability of results difficult as these can have a significant effect on recovery.

Most notably, the virus was unable to be isolated from any blood specimens and only 5.4% of saliva specimens. The lack of success in isolating the virus from blood is interesting given the extended detection of viral RNA in blood specimens. As all 33 attempts using blood were cultured with Vero or C6/36 cells, the difficulty in isolating the virus from blood does not appear to be related to cell line. This could indicate that molecular methods are only detecting remnants of the virus as opposed to viable RNA. There could also be a potential inhibitor in whole blood that makes viral isolation difficult.

Some of the difference between viral clearance estimates may be due to differences in analytical methodologies. These differences may also be due to limitations in utilizing data derived from a systematic review of the literature. Although this systematic review builds upon the earlier work conducted by Lessler and colleagues, the data presented across the wide variety of records reviewed was highly variable in its presentation and quality. For example, data were only available in 11.7% of the 792 cases to be able to determine the incubation period, potentially indicating a bias that could influence the resulting estimate. Similarly, availability of rRT-PCR results was often unavailable, likely due to lack of routine serial testing, potentially limiting the accuracy of viral clearance estimates. These inconsistencies indicate a need for a uniform method for reporting pertinent and complete data for case reports and case series of infectious with infectious diseases. Sample size was also limited for certain specimen types, especially CSF and vaginal specimens. The uneven distribution of specimen types also reflects the use of serum in serological testing and, potentially, the paradigm that serum is the specimen of choice for diagnosing ZIKV infection. This study is also limited by the

variability in diagnostic testing methodologies; the varying sensitivities of rRT-PCR assays could potentially impact their ability to detect viral RNA, especially as viral load decreases. These estimates can only be applied to symptomatic cases of ZIKV disease as asymptomatic cases were not included. Lastly, these viral clearance estimates must be interpreted cautiously, as they were measured using rRT-PCR methods and do not necessarily reflect viability of the virus to infect other cells. This is helpful for establishing diagnostic criteria, but not necessarily in determining if a patient is still infectious.

Understanding the natural history of infections with ZIKV is critical to controlling its spread in communities. Knowing the virus' incubation period allows clinicians and public health practitioners to better assess potential exposure to the virus. Insight into the rate of viral clearance in various specimen types also aids in the correct application and interpretation of test results. Testing algorithms, especially for molecular assays, should take into account the variability observed in different specimen type to maximize detection of infections with ZIKV. This is especially important given the poor ability of current serological assays to discriminate between different *Flaviviridae* infections. The different rate of viral clearance between serum and blood specimens may also have implications for the donation of different blood components.

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Chapter 4

Characterization of Viral Load

Background

The worldwide outbreak of ZIKV disease beginning in 2015 was challenging for both clinicians and public health agencies because the nonspecific and often absent symptoms associated with the virus made detecting infections particularly difficult. Up to 80% of infections are thought to be asymptomatic (1, 2), and even when symptoms present, they are not pathognomonic and usually mild in nature (3). This makes the initial detection of the disease difficult and potentially allows the virus to establish itself in a community prior to detection (4-6). Further compounding the issue is the low viral load observed in routine clinical specimens, such as serum and urine (7-11). Serological methods, including the detection of IgM against ZIKV or PRNT for ZIKV, are also complicated by the cross-reactivity of the virus with other flaviviruses, especially DENV (1, 12). This makes diagnosis by serological methods challenging and, often, results in an inconclusive or unconfirmed diagnosis (12, 13). Given the emergence of ZIKV as a public health threat, diagnostic assays that can fully overcome these obstacles have not yet been developed (14). Understanding the viral load in easily obtainable clinical specimens can help optimize the development and utilization of such assays.

Viral load can be quantitated using rRT-PCR assays. These assays directly detect the genetic material of the pathogen in the patient's specimen. For true quantitation of viral load with these tests, an internal control or standard must be added to the specimen and amplified alongside the pathogen's DNA or RNA. Viral load testing has become a standard of practice for monitoring the progression of and providing treatment for both

human immunodeficiency virus and hepatitis C virus. When an internal standard is not used, the rRT-PCR test is generally referred to as a semi-quantitative assay. The resulting cycle threshold (C_t) value is an indication of the amount of virus in the specimen. C_t values have recently been used to monitor the progression of Ebola virus disease in patients and predict patient outcomes (15-18) . Semi-quantitative rRT-PCR has also been used in the recent outbreak of the severe acute respiratory syndrome coronavirus 2 in China to monitor the progression of the virus (19) .

The dramatic increase in the number of cases of Zika virus disease seen since 2015 has given researchers an opportunity to learn more about this once rare virus and the disease it causes. As part of its routine surveillance activities, the Florida DOH investigates reports of potential cases of ZIKV infection in Florida. From 2016 to 2017, the Florida DOH identified 1,733 cases of ZIKV infection (20), including those that were travel-associated and those that were locally acquired. As part of these investigations, epidemiological data were gathered and diagnostic specimens tested, providing valuable, high fidelity data that can provide insight into the course of the disease. Herein, we utilize these data to estimate the progression of viremia and the time to viral clearance in in whole blood, serum, and urine specimens.

Methods

Confirmed and probable cases of non-congenital ZIKV infection identified from 2016 to 2017 through testing performed at one of the three locations of the Florida DOH's Bureau of Public Health Laboratories (BPHL), were included in this study. Although the case definition for ZIKV infection changed over the course of the response,

the case definition typically included some variation of clinically compatible disease, complication of pregnancy, or neurologic symptoms, and laboratory evidence supporting the diagnosis of ZIKV infections (21). Confirmed cases were those meeting general epidemiological criteria with either unambiguous serological results (positive IgM ELISA, positive neutralizing antibody titers against ZIKV, and negative neutralizing antibodies against DENV) or RNA detected by rRT-PCR. Probable cases were those meeting epidemiological criteria with no positive rRT-PCR result and ambiguous serological results (22). In this analysis, both travel-associated and locally acquired cases were included. Asymptomatic and congenitally acquired cases were excluded as no symptom onset date could be determined. This study was reviewed and approved by both the Florida DOH and Florida International University (FIU) Institutional Review Boards.

Clinical and epidemiological data were obtained from Merlin, the Florida DOH's surveillance database. The data utilized in this study included gender, age, pregnancy status, YFV vaccination status, case status (confirmed or probable), and symptoms, including fever, rash, headache, joint pain, conjunctivitis, and muscle pain. Clinical and epidemiological data were typically self-reported to investigators. Corresponding laboratory results were obtained from the BPHL's laboratory information system (LIS) and assay run reports. Laboratory variables included specimen type; days collected post-symptom onset (DCPSO, calculated by subtracting the date of symptom onset from the date of specimen collection); type of rRT-PCR assay; and C_t value. Both sets of data were linked by matching Merlin and LIS identifiers and then de-identified for analysis.

Specimens were tested according to the testing algorithm in place at the time of collection. Molecular detection of ZIKV was accomplished through one of two rRT-PCR

assays, a laboratory-developed test (LDT) previously described (7) and the CDC's Trioplex rRT-PCR Assay (23). The former was primarily used at two of the BPHL locations during 2016 before transitioning to the Trioplex Assay in 2017; the third BPHL location used the Trioplex Assay exclusively. Although three primer and probe sets for three separate RNA targets were utilized with the LDT, only one target was utilized when comparing C_t values as it matched the target of the Trioplex Assay. Further, the LDT was always performed in duplicate; whereas the Trioplex Assay was performed in singlicate. When two C_t values were reported, the mean of the two values was utilized for this study; if only one of the replicates demonstrated amplification, the single value was used. In practice, each rRT-PCR assay has an established cutoff value and is typically reported as detected, not detected, or equivocal. In this study, however, C_t values were analyzed without regard to these cutoff values as this represents amplification of the viral target.

Descriptive analysis of the study population and laboratory results was conducted. Dataset characteristics were compared using the Chi-square Test or Fisher's Exact Test, where appropriate, using a significance level of 0.05. A logistic regression model was created to predict the detection of ZIKV RNA by rRT-PCR (any specimen with a C_t value without regard to the cutoff value), the dependent variable, and identify factors associated with the clearance of viral RNA. The independent variables included specimen type, DCPSO, age, gender, and number of key symptoms. Pregnancy status was assessed through a second logistic model that only included females. Independent variables with an adjusted odds ratio (aOR) 95% confidence interval (95% CI) including 1.00 were excluded from the final model. In order to account for repeated measures due to repeated sampling (multiple specimens collected on multiple days post-symptom onset) on some,

but not all, cases, PROC GENMOD with the logit link function and Bonferonni correction for multiple comparisons were utilized for this analysis. Statistical analyses were performed using software using SAS software version 9.4 (Cary, NC, USA).

Results

A total of 2,044 specimens tested by rRT-PCR, representing 934 cases, were included in this study. ZIKV RNA was detected in 75.7% (707) of these cases (Table 1). ZIKV RNA was detected in at least one urine specimen in 61.6% (575) of cases, in at least one serum specimen in 44.4% (415) of cases, and in at least one whole blood specimen in 3.3% (31) of cases (data not shown). As expected, given the importance of rRT-PCR in the case definition ZIKV, the majority of included cases (824, 88.2%) were confirmed as ZIKV infections (Table 1). Of the 934 cases, 535 (57.3%) were identified in females. The mean ages of females and males were 40.1 and 41.3 years, respectively. Of the 535 women included in this study, 32 (6.0%) were pregnant at the time of detection (Table 1). The most commonly reported symptom was rash (93.6%; 874). Of cases, 74 (7.0%) reported being vaccinated against YFV, and 282 (30.2%) were unaware of their vaccination status. The median DCPSO for females was 5 days versus 4 days for males.

Table 1. Demographic and clinical characteristics of patients with confirmed and probable ZIKV infection.

	Female n (%)	Male n (%)	Total n (%)	
Total	535 (57.3)	399 (42.7)	934 (100.0)	p-value*
Case status				0.024
Confirmed	461 (86.2)	363 (91.0)	824 (88.2)	
Probable	74 (13.8)	36 (9.0)	110 (11.8)	
RNA detected in at least one specimen				0.091
Yes	394 (73.6)	313 (78.4)	707 (75.7)	
No	141 (26.4)	86 (21.6)	227 (24.3)	
Specimen detection†				0.312
Serum	232 (41.1)	183 (40.1)	415 (58.7)	
Urine	320 (56.6)	255 (55.9)	575 (81.3)	
Whole blood	13 (2.3)	18 (3.9)	31 (4.4)	
Pregnancy status				
Pregnant	32 (6.0)	-	32 (6.0)	
Not pregnant	503 (94.0)	-	503 (94.0)	
Age (years)				0.266
Mean	40.1	41.3	40.6	
Range	1-81	1-86	1-86	
0-4	3 (0.6)	1 (0.3)	4 (0.4)	
5-9	7 (1.3)	5 (1.3)	12 (1.3)	
10-19	32 (6.0)	31 (7.8)	63 (6.8)	
20-29	107 (20.0)	56 (14.0)	163 (17.5)	
30-39	131 (24.5)	94 (23.6)	225 (24.1)	
40-49	101 (18.9)	84 (21.1)	185 (19.8)	
50-59	88 (16.5)	81 (20.3)	169 (18.1)	
≥60	66 (12.3)	47 (11.8)	113 (12.1)	
Symptoms‡				
Rash	509 (95.1)	365 (91.5)	874 (93.6)	0.024
Fever	342 (63.9)	309 (77.4)	651 (69.7)	<0.001
Arthralgia	345 (64.5)	252 (63.2)	597 (63.9)	0.676
Conjunctivitis	234 (43.7)	163 (40.9)	397 (32.2)	0.377
Myalgia	167 (31.2)	134 (33.6)	301 (32.2)	0.444
Headache	163 (30.5)	124 (31.1)	287 (30.7)	0.842
Number of key symptoms				0.098
Zero	1 (0.2)	0 (0.0)	1 (0.1)	
One	30 (5.61)	16 (4.0)	46 (4.9)	
Two	91 (17.0)	79 (19.8)	170 (18.2)	
Three	202 (37.7)	120 (30.1)	322 (34.5)	
Four	130 (24.3)	117 (29.3)	247 (26.5)	

Five	64 (12.0)	57 (14.3)	121 (13.0)	
Six	17 (3.2)	10 (2.5)	27 (2.9)	
YFV§ vaccination ever				0.035
Vaccinated	38 (7.1)	36 (9.0)	74 (7.9)	
Not vaccinated	350 (65.4)	228 (57.1)	578 (61.9)	
Unknown	147 (27.5)	135 (33.8)	282 (30.2)	
Days collected post-symptom onset				<0.001
Total specimens	1,208 (59.1)	836 (40.9)	2,044 (100.0)	
Range	-4 to 300	-4 to 267	-4 to 300	
Mean	20.1	10.2	16.1	
Median	5	4	5	
<0	2 (0.2)	6 (0.7)	8 (0.4)	
0-3	425 (35.2)	325 (38.9)	750 (36.7)	
4-7	307 (25.4)	252 (30.1)	559 (27.4)	
8-11	95 (7.9)	63 (7.5)	158 (7.7)	
12-15	57 (4.7)	51 (6.1)	108 (5.3)	
16-19	41 (3.4)	25 (3.0)	66 (3.2)	
20-23	37 (3.1)	29 (3.5)	66 (3.2)	
24-27	37 (3.1)	18 (2.2)	55 (2.7)	
≥28	207 (17.1)	67 (8.0)	274 (13.4)	

*Chi-square test for all comparisons except number of key symptoms and days collected post-symptom onset (Fisher's Exact Test); significance level of 0.05.
†by rRT-PCR
‡ Cases may have more than one symptom; percentages will not add up to 100
§Yellow fever virus

Of the 2,044 collected specimens, 1,208 (59.1%) were collected from females (Table 2). Serum was the specimen collected most frequently (1,052; 51.5%), followed by urine (904; 44.2%) and whole blood (88; 4.3%) (Table 2). Specimens were collected between -4 and 300 days with almost two-thirds of specimens (1,317; 64.4%) collected within a week of symptom onset (Table 1).

Table 2. rRT-PCR results by specimen type and gender.

	Female	Male	Total	p-value*
Serum				0.197
Detected	262 (41.5)	191 (45.5)	453 (43.1)	
Not detected	370 (58.5)	229 (54.5)	599 (56.9)	
Total	632 (60.1)	420 (39.9)	1,052 (100.0)	
Urine				0.003
Detected	333 (62.4)	266 (71.9)	599 (66.3)	
Not detected	201 (37.6)	104 (28.1)	305 (33.7)	
Total	534 (59.1)	370 (40.1)	904 (100.0)	
Whole blood				0.573
Detected	14 (33.3)	18 (39.1)	32 (36.4)	
Not detected	28 (66.7)	28 (60.9)	56 (63.6)	
Total	42 (47.7)	46 (52.3)	88 (100.0)	
Total				0.004
Detected	609 (50.4)	475 (56.8)	1,084 (53.0)	
Not detected	599 (49.6)	361 (43.2)	960 (47.0)	
Total	1,208 (59.1)	836 (40.9)	2,044 (100.0)	

*p-values determined conducting univariate regression between gender and detection outcome stratifying by specimen type

ZIKV RNA was detected in 53.0% of all specimens (Table 3) with ZIKV RNA being detected most often in urine specimens (66.3%), followed by serum (43.1%), and whole blood (36.4%). ZIKV RNA was detected in 71.9% of male urine specimens as compared to 62.4% of female urine specimens (p = 0.003) (Table 2). Of those cases where both serum and urine were collected on the same day (n = 708), ZIKV RNA was detected in both serum and urine in 277 (39.1%) cases; in only urine in 257 (36.3%) cases; in only serum in 47 (6.6%) cases and not detected in either specimen type in 127

(17.9%) cases (Table 4). For each DCPSO group except <0, ZIKV was detected in urine at a higher frequency than in serum.

Table 3. ZIKV RNA detected by specimen type and days collected post-symptom onset.

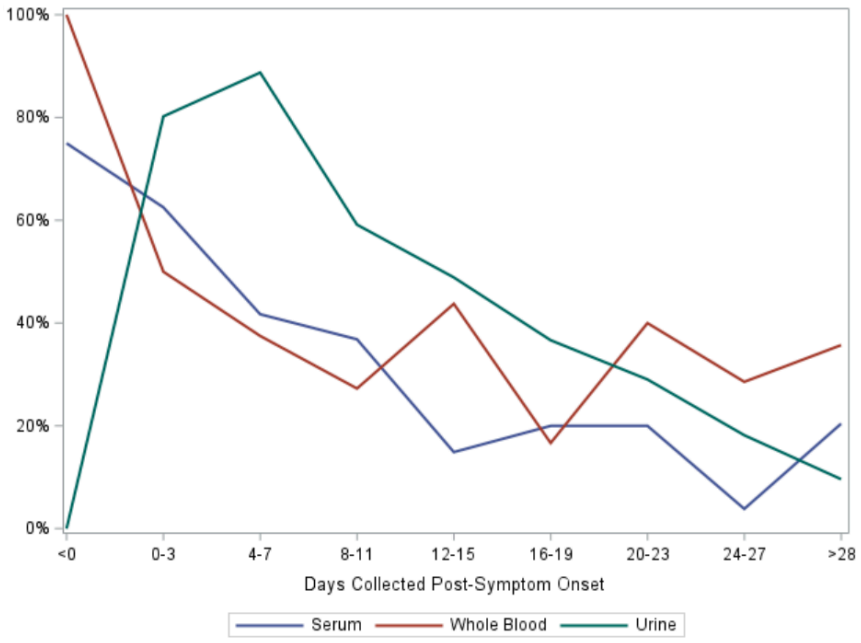
Days collected post-symptom onset	Serum			Urine			Whole blood			Total		
	Tested	Detected	%	Tested	Detected	%	Tested	Detected	%	Tested	Detected	%
<0	4	3	75.0	3	0	0.0	1	1	100.0	8	4	50.0
0-3	395	247	62.5	349	280	80.2	6	3	50.0	750	530	70.7
4-7	302	126	41.7	249	221	88.8	8	3	37.5	559	350	62.6
8-11	76	28	36.8	71	42	59.2	11	3	27.3	158	73	46.2
12-15	47	7	14.9	45	22	48.9	16	7	43.8	108	36	33.3
16-19	30	6	20.0	30	11	36.7	6	1	16.7	66	18	27.3
20-23	30	6	20.0	31	9	29.0	5	2	40.0	66	17	25.8
24-27	26	1	3.8	22	4	18.2	7	2	28.6	55	7	12.7
≥28	142	29	20.4	104	10	9.6	28	10	35.7	274	49	17.9
Total	1,052	453	43.1	904	599	66.3	88	32	36.4	2,044	1,084	53.0

Table 4. Comparison of ZIKV RNA detection amongst 708 paired serum and urine specimens collected on the same day post-symptom onset.

Day collected post symptom onset	Pairs tested n	Serum detected n (%)	Urine detected n (%)	Serum only detected n (%)	Urine only detected n (%)	Neither detected n (%)	Both detected n (%)
<0	2	2 (100.0)	0 (0.0)	2 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)
0-3	303	186 (61.4)	265 (87.5)	27 (8.9)	106 (35.0)	11 (3.6)	159 (52.5)
4-7	231	100 (43.3)	205 (88.7)	7 (3.0)	112 (48.5)	19 (8.2)	93 (40.3)
8-11	45	13 (28.9)	32 (71.1)	2 (4.4)	21 (46.7)	11 (24.4)	11 (24.4)
12-15	27	5 (18.5)	13 (48.1)	1 (3.7)	9 (33.3)	13 (48.1)	4 (14.8)
16-19	17	3 (17.6)	5 (29.4)	1 (5.9)	3 (17.6)	11 (64.7)	2 (11.8)
20-23	16	5 (31.3)	6 (37.5)	1 (6.3)	2 (12.5)	9 (56.3)	4 (25.0)
24-27	10	0 (0.0)	1 (10.0)	0 (0.0)	1 (10.0)	9 (90.0)	0 (0.0)
≥28	57	2 (3.5)	7 (12.3)	6 (10.5)	3 (5.3)	44 (77.2)	4 (7.0)
Total	708	314 (44.4)	534 (75.4)	47 (6.6)	257 (36.3)	127 (17.9)	277 (39.1)

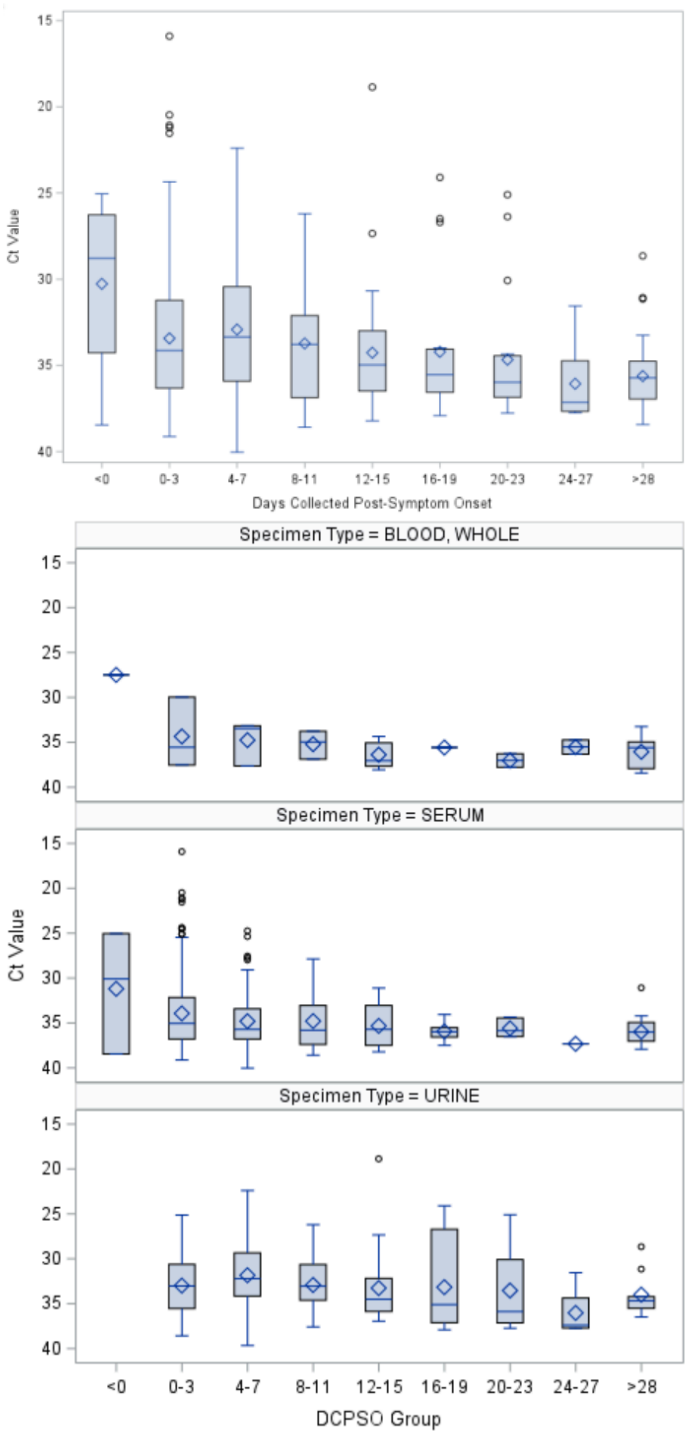
Almost half (48.9%) of all positive specimens were collected within three days of symptom onset (Table 3). Among specimens collected at 4 weeks or greater, 49 (17.9%) had detectable ZIKV RNA. At the extreme, ZIKV RNA was detected in a serum specimen at 127 DCPSO, urine at 64 DCPSO, and whole blood at 94 DCPSO. The detection of ZIKV RNA within serum appears to peak before or near the onset of symptoms and declines thereafter; whereas the detection of ZIKV RNA within urine appears to peak near the end of the first week after symptom onset and then steadily declines. Although the sample size is much smaller, the detection of ZIKV RNA within whole blood appears to remain relatively steady over most of the DCPSO groups (Figure 1). Of the positive whole blood specimens, 10 (31.3%) were collected ≥28 days post-symptom onset. Boxplots of C_t values by each DCPSO group also indicate the decreasing trend of viral load over time (Figure 2).

Figure 1. Proportion of specimens with ZIKV RNA detected* over time.



*Detection is defined as any amplification above the threshold

Figure 2. Boxplot of C_t values* by days collected post-symptom onset group



*C_t values are inversely proportional to viral load.
DCPSO: days collected post-symptom onset

Of the 1,084 specimens with detectable ZIKV RNA, 1,082 had C_t values available, allowing viral load to be assessed by proxy. Visualization and normality testing of C_t values indicated that the distribution of C_t values is not normal (Figure 3), necessitating the use of non-parametric tests. The median C_t values of serum, urine, and whole blood were 35.3 (IQR = 3.8), 32.9 (IQR = 5.0), and 35.6 (IQR = 2.6), respectively (Table 5). The highest and lowest C_t values observed were 15.9 and 40.0, respectively, both in serum. Notably, the lowest C_t value observed in whole blood was 27.5, collected the day before symptom onset. The range of urine C_t values appears to be similar to that of serum.

Figure 3. Distribution of Ct values from A) all 1,082 specimens with detectable ZIKV RNA, B) serum specimens only, C) urine specimens only, and D) whole blood specimens only.

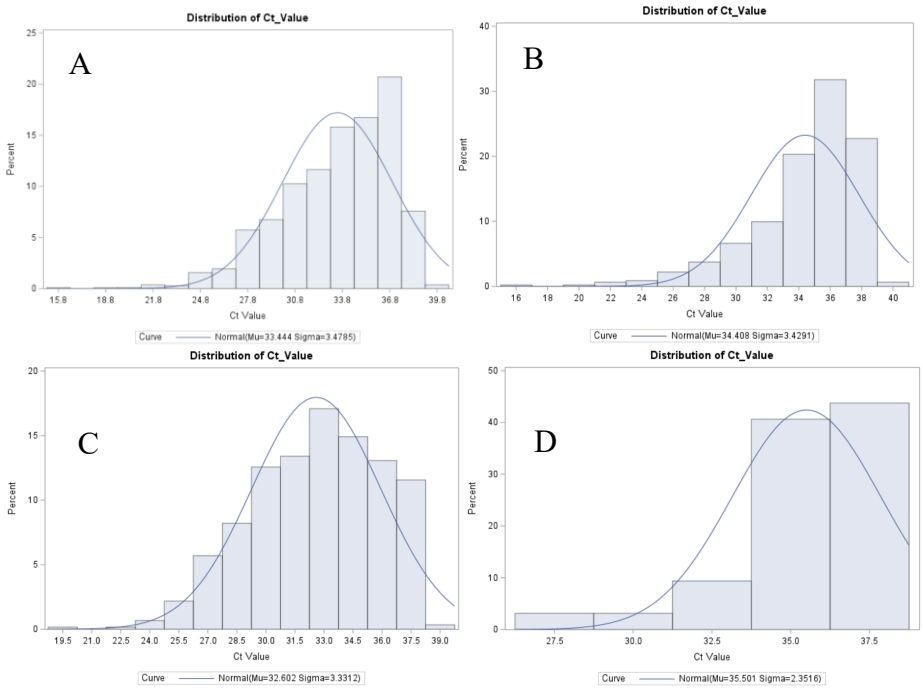


Table 5. Comparison of median C_t values of specimens with detectable RNA.

	n	Median C_t value	Interquartile Range	Range
Total				
Gender				
Female	608	34.2	5.2	20.5 to 38.6
Male	474	34.0	4.9	15.9 to 40.0
Age group				
≤ 18 years	74	34.5	3.8	26.2 to 39.0
>18 years	1,008	34.1	5.2	15.9 to 40.0
Specimen type				
Serum	453	35.3	3.8	15.9 to 40.0
Urine	597	32.9	5.0	18.9 to 39.7
Whole blood	32	35.6	2.6	27.5 to 38.4
Pregnancy status				
Pregnant	72	35.2	3.8	27.4 to 38.6
Not pregnant	536	33.9	5.3	20.5 to 38.6
YFV* vaccination status				
Vaccinated	93	34.2	4.7	22.4 to 38.2
Not vaccinated	668	34.2	5.1	20.5 to 39.7
Unknown	321	33.8	5.4	15.9 to 40.0
Symptoms				
Rash present	1,037	34.1	5.1	15.9 to 40.0
Rash absent	45	34.2	6.3	21.2 to 37.8
Fever present	782	34.2	5.2	15.9 to 39.7
Fever absent	300	34.0	4.9	25.2 to 40.0
Arthralgia present	675	33.9	5.3	20.5 to 39.7
Arthralgia absent	407	34.4	4.9	15.9 to 40.0
Conjunctivitis present	486	33.9	5.3	18.9 to 40.0
Conjunctivitis absent	596	34.3	4.9	15.9 to 39.7
Myalgia present	364	34.1	5.2	18.9 to 39.7
Myalgia absent	718	34.1	5.1	15.9 to 39.7
Headache present	341	33.9	5.0	20.5 to 39.7
Headache absent	741	34.2	5.2	15.9 to 40.0
Number of key symptoms				
One	35	34.5	4.5	25.4 to 37.4
Two	185	33.7	5.4	15.9 to 40.0
Three	381	34.5	4.6	24.5 to 38.6
Four	293	34.0	5.3	18.9 to 39.1
Five	151	33.5	5.8	21.1 to 39.7
Six	35	33.7	5.2	20.5 to 38.1

*Yellow Fever Virus

There does not appear to be a difference in median C_t value between specimens collected from females and males or between specimens collected from those 18 years old or younger and those older than 18 (Table 5). Median C_t values of the specimens from pregnant (n = 72) and non-pregnant (n = 536) women appear to be lower among nonpregnant women (35.2 versus 33.9). This appears to be due to the extended sampling conducted on pregnant women. When only specimens collected within three weeks were compared, there was no significant difference (p = 0.704). Conversely, the median C_t values of specimens from those that were previously vaccinated against YFV (n = 93), those that were not vaccinated (n = 668), and those with an unknown vaccination status (n = 321) did not appear to be different. Median C_t values by symptom presentation all appear to be fairly similar, showing no more than 0.5 C_t difference amongst any of the groups. Similarly, no differences were observed based on the total number of symptoms reported.

The logistic regression model confirmed the significance of specimen type on the detection of ZIKV RNA (Table 6). The final model included specimen type (whole blood, serum, and urine), DCPSO, age, and an interaction effect between specimen type and DCPSO. The adjusted odds of detection in urine was significantly greater than in whole blood (aOR = 8.04, 95% CI 4.34 to 14.87). The adjusted odds of detection was slightly greater in serum than in whole blood (OR = 1.51, 95% CI 0.84 to 2.72), but was not statistically significant. Age, gender, and key symptoms were found to be insignificant and not included in the final model (data not shown). Within the females only model, pregnancy was also found to be a significant indicator for the detection of ZIKV RNA (aOR = 3.29, 95% CI 1.89 to 5.74). To assess for a bias caused by extended

sampling of pregnant women with respect to DCPSO, the analysis was limited to specimens collected at three weeks or less (as noted above), and similar results were observed (aOR = 3.33, 95% CI 1.62 to 6.81; data not shown). DCPSO and the interaction effect between DCPSO and specimen types were included in the final model as the Type 3 analysis of effects showed significance. The interaction between urine and DCPSO was significant (aOR = 0.91, 95% CI 0.88 to 0.94), indicating that the odds of detection in urine decrease as DCPSO increases. To confirm the effect of DCPSO on specimen type, logistic regression was conducted to predict detection using DCPSO alone on each individual specimen type. Urine (OR = 0.90, 95% CI 0.88 to 0.93) and serum (OR = 0.97, 95% CI 0.95 to 0.99) were found to be significant but whole blood (OR = 0.99, 95% CI 0.98 to 1.00) was not. In other words, the rate of viral clearance in urine and serum appear to occur more rapidly than in whole blood.

Table 6. Adjusted odds ratios for the final logistic regression model (including specimen type, DCPSO, pregnancy status†, and the interaction between DCPSO and specimen type) for the detection of ZIKV RNA by rRT-PCR.

Variable	aOR	95% Confidence Interval	p-value*
Specimen type			<0.001
Whole blood	Referent	Referent	Referent
Serum	1.51	0.84 to 2.72	0.165
Urine	8.04	4.34 to 14.97	< 0.001
DCPSO†	0.99	0.98 to 1.00	0.220
Age	1.01	1.00 to 1.02	0.029
Pregnant‡	3.29	1.89 to 5.74	< 0.001
Interaction with DCPSO			0.001
Whole blood	Referent	Referent	Referent
Serum	0.98	0.96 to 1.00	0.075
Urine	0.91	0.88 to 0.94	< 0.001

*Bonferroni-adjusted alpha level = 0.017
†Days collected post-symptom onset
‡Pregnancy determined from females-only model

Discussion

Detection of viral RNA is the ideal method to conclusively identify acute infection with ZIKV. Specimen selection and timing of collection is critical to maximize success in detecting ZIKV RNA. In our dataset, ZIKV RNA was most frequently detected in urine specimens (66.3%). ZIKV RNA was detected in urine at the highest frequency of all three specimen types through 19 days post-symptom onset, allowing for the detection of infection well after the onset of symptoms. ZIKV RNA was detected in serum in almost two-thirds of specimens collected within 3 days of symptom onset, but the frequency of detection decreased rapidly thereafter. This is comparable to what has been reported in New York State (10, 24). However, ZIKV RNA was detected at a lower rate in serum alone than in New York (6.6% versus 26.2%). In fact, within paired

specimens, ZIKV RNA was detected in urine alone at nearly six times the frequency as serum alone in our dataset. This difference may be due in part to expanded testing of pregnant women, leading to a wider specimen collection window, and the utilization of systematic surveys, resulting in a potential bias towards urine specimens as corresponding sera were not collected.

The timing of specimen collection is also critical to maximize the likelihood of detection of ZIKV. Within the first 4 weeks post-symptom onset, the frequency of detection of ZIKV RNA in serum was always lower than that of urine. By the beginning of the second week post-symptom onset, the frequency of detection within serum specimens decreased by almost half. ZIKV RNA was detected most frequently in urine specimens. Overall, however, it appears that ZIKV RNA may remain detectable in whole blood longer over time. Almost a third of positive whole blood specimens were collected ≥28 days post-symptom onset. These observations concur with previous studies (25-27) and highlight the importance of specimen selection to maximize the detection of ZIKV RNA in relation to the timing of specimen collection.

Urine specimens demonstrated the highest viral load overall, as indicated by median C_t value. This coincides with the increased detection of ZIKV RNA in urine specimens as compared to other specimen types. In a previous study, the viral loads of urine specimens were observed to be a log higher than those of serum specimens (10) but were observed to be lower in a separate pediatric cohort (28). A study by Judice and colleagues (29) compared viral loads between urine and whole blood specimens, finding that the viral load in urine was higher than that of whole blood specimens. No correlation was seen between any of the six key symptoms and the viral load of specimens,

corresponding with a report from Musso and colleagues (11). These estimates, as in our study, were determined using rRT-PCR methods and may not truly reflect intact virus.

These findings are important when considering appropriate testing strategies for ZIKV. Current guidance for rRT-PCR requires paired specimens when testing any specimen type other than serum, meaning serum must always be collected at the same time as urine or other specimens, if they are to be tested. Although this is due in part to the importance of serological diagnosis of ZIKV, it also contributes to a paradigm that serum is the single most important specimen type for rRT-PCR and potentially prohibits testing when serum is not collected along with urine, whole blood, or other specimen types. In fact, all 11 rRT-PCR assays currently approved for emergency use in the United States and able to test specimens other than serum or plasma require matched specimens (30). The experiences of the FDOH also challenge the testing algorithms currently recommended by the CDC. These guidelines suggest that rRT-PCR testing should be limited to specimens collected within 7 days of symptom onset(31). As demonstrated in this study, ZIKV RNA can be detected in approximately a quarter of all specimens collected in the first three or so weeks following symptom onset. Although low, this could potentially provide unambiguous evidence for ZIKV that may be missed due to limitations of serological testing. This, of course, must be balanced with the number of negative results that may be encountered as a result of a wider specimen collection window.

This study is limited to symptomatic infections with ZIKV. Therefore, it is impossible to assess complete performance of the assay or the implications of widening the specimen collection window. Symptom onset date was self-reported, and due to the

generally mild nature of ZIKV infection, it is possible that patients could not accurately recall the onset date, which would affect the accuracy of the DCPSO calculation as well as viral load estimates and characterization of ZIKV detection frequency. However, patient were was systematically collected by trained interviewers. The small number of whole blood specimens tested (only 4.3% of all specimens in this dataset) limits the ability to draw conclusions on the detection and viral load of those specimens. Viral load was estimated using C_t values produced by rRT-PCR testing and detects viral RNA, not intact virus. These values serve as a proxy for viral load as they were not quantified using a true standard curve and internal control and are therefore prone to variation. This variation can come from operator training and error, degradation in reagents, pipetting error, and other sources. Further studies should assess viral load through standardized quantitative rRT-PCR or other accepted methods. Any method relying on rRT-PCR is limited in that it does not necessarily indicate if the detected virus is viable or not since it only detects viral RNA.

With the finite amount of laboratory resources to test these specimens, optimizing specimen collection and testing algorithms is necessary. Understanding the progression of virus and viral load in various specimen types is critical to refining testing strategies for ZIKV. Effective utilization of rRT-PCR can assist in limiting the number of specimens requiring IgM or PRNT for confirmation, both of which require increased time and resources, are not as readily available as rRT-PCR, and often do not provide confirmation as intended. Increased reliance on rRT-PCR should allow providers, patients, and investigators to receive results quicker and potentially provide for fewer unambiguous results.

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Chapter 5

Evaluation of Testing Guidelines

Background

In the summer of 2016, the Florida DOH detected the first cases of mosquito-borne, locally transmitted ZIKV infections in the continental United States in Miami-Dade and Broward Counties (1, 2). This discovery prompted the mobilization of public health and mosquito control resources in an effort to rapidly contain the outbreak and protect the health of the state's residents and visitors (3). As part of this response, the Florida DOH's Bureau of Epidemiology (BOE) and BPHL, along with county health departments, were responsible for investigating suspected cases and testing specimens.

Because of the nonspecific, often absent, symptoms of ZIKV infections (4), laboratory testing plays a critical role in identifying cases of infections caused by ZIKV. The most commonly available assays for ZIKV are rRT-PCR tests, which detect the nucleic acid of the virus directly, and IgM ELISA tests, which identify the host's antibody response to a recent infection. A third test, the PRNT, is used to measure the titer of neutralizing antibodies against the virus (5). Because of the clinical similarities between infections, DENV testing is often ordered along with ZIKV tests. However, interpretation of serological assay (IgM and PRNT) results can be complicated by the cross-reactivity with different flaviviruses (5-8). Similarly, the viral load associated with ZIKV infections has been reported to be substantially lower than that associated with other flaviviruses (6, 9) and has been observed to be lower in serum than urine (10). Both of these factors impact how and when these assays should be used to maximize the probability of detection while limiting the opportunity for discrepant or unreliable results.

The CDC provides extensive guidance for testing patients for ZIKV (11) and updated these guidelines in 2019 to incorporate testing for DENV (12). The intent of this guidance is to maximize the likelihood of detecting cases while ensuring resources are not needlessly expended. This is further complicated by the high proportion of asymptomatic cases and the risk presented to the fetuses of pregnant women (13, 14). Three separate algorithms are recommended by the CDC for individuals with possible exposure: non-pregnant symptomatic individuals, pregnant symptomatic women, and pregnant asymptomatic women. Exposures can include living in or traveling to an area with risk of ZIKV or unprotected sexual contact with someone at risk of exposure. These algorithms have been refined as more is learned about the course of infection. Two significant updates included in the 2019 algorithms include narrowing the specimen collection window for rRT-PCR to ≤7 days and including testing for IgM against DENV. The latter significantly increases the number of potential results given by the algorithm. The 2019 update also includes testing specimens by DENV rRT-PCR, but this is not addressed here.

Testing during the ZIKV outbreak in Florida was primarily provided by commercial laboratories, BPHL, and the CDC (15). Positive specimens from commercial laboratories were referred to BPHL for confirmation. The BPHL is composed of three public health laboratories located in Jacksonville, Miami, and Tampa. The CDC initially performed all PRNT testing and provided surge capacity for rRT-PCR and ZIKV IgM testing during periods of increased volume. The Florida DOH generally relies on the CDC's guidance, but also significantly expanded the availability of free testing to pregnant women following the identification of autochthonous transmission within the

state (3). Testing conducted by BPHL was also generally expanded to include serological testing for DENV (both IgM and IgG) due to the large population of foreign-born residents that may have been exposed to other arboviruses, such as DENV in their country of birth or during travel. Although testing for DENV IgM is suggested in the 2019 CDC ZIKV testing guidance, this was not generally recommended during the outbreak. According to the 2016 American Community Survey, an estimated 19.9% of Florida's population was born outside of the United States (16). In Miami-Dade County, where local transmission of ZIKV occurred, approximately 52.2% of the population was foreign-born. This additional testing for DENV provides critical data on the effectiveness of the serological assays and allows for retrospective testing of the 2019 updated guidance.

Understanding how these assays perform is also important when investigating potential cases of the disease and determining whether cases were acquired locally, as a result of travel to an endemic area, or acquired through sexual contact. In addition to these concerns, all three assay types have different demands in terms of complexity, cost, availability, and turnaround time, requiring laboratories and epidemiologists to optimize testing algorithms. Using the assay results and epidemiological data from the Florida DOH, we retrospectively compare the efficacy of the CDC's 2017 and 2019 testing strategies for ZIKV in the context of locally acquired and travel-associated cases of the virus from 2016 and 2017. With the shortening of the specimen collection period, we expect that laboratory testing is able to confirm significantly fewer infections.

Methods

Dataset. Cases were identified through both active and passive surveillance previously described (1, 3, 17). All confirmed and probable cases of ZIKV infection identified between 2016 and 2017 by the Florida DOH were included in this study except those acquired congenitally. Generally, confirmed cases are those meeting general epidemiological criteria, such as residing in or having recent travel to an area with known ZIKV transmission, and confirmatory laboratory evidence, such as direct detection of viral RNA or both a positive PRNT (titer ≥ 10) against ZIKV and a negative PRNT (titer <10) against DENV. Generally, probable cases are those meeting epidemiological criteria with presumptive laboratory evidence, such as a positive ZIKV IgM result with an ambiguous PRNT result (18). Both symptomatic and asymptomatic cases were included. Qualitative molecular and serological laboratory results included in this analysis were performed by either the BPHL or the CDC; no results from commercial laboratories are included as they were repeated at the BPHL. Ten specimens were excluded as they were collected before symptom onset; two specimens collected greater than a year post-symptom onset were excluded. Clinical and epidemiological data were collected through epidemiological investigations, chart review, and laboratory requisitions. All data were collected as part of routine public health investigations and were stored in the Florida DOH's disease surveillance system. Data on country of birth were not collected and, therefore, not included in the analysis. Laboratory data were matched to case data by matching case and specimen identifiers to collection dates. Where discordant results for a single specimen and collection dates were reported (e.g., if a specimen test was repeated), the more conclusive result was included. For example, if both an indeterminate and

positive result were available for the same specimen, the positive result was used in the analysis as the assay would have been repeated to resolve the indeterminate result. For algorithm evaluation, equivocal, indeterminate, and inconclusive results were treated as negative results as these specimens would be further tested in an actual investigation. Both case-level determinations (e.g., acute infection with ZIKV or infection with flavivirus) and individual laboratory results are presented. In the context of algorithm evaluation, cases are adjudicated by meeting particular testing criteria outlined by the appropriate testing algorithm. Cases were de-duplicated according to hierarchical value of the algorithm result. Broadly, this hierarchy was prioritized from acute infections, infections of undetermined timing, presumptive infections, no evidence of infection, and those with no algorithm result. Protocol approval was granted by both the Florida DOH and FIU Institutional Review Boards.

Analysis. Descriptive analysis of the dataset was conducted with SAS 9.4. Case- and specimen-level data were used to compare the 2017 and 2019 CDC testing recommendations (11, 12). Specimens were first categorized according to symptom presentation (symptomatic or asymptomatic) and pregnancy status to determine with which CDC algorithm (non-pregnant symptomatic, pregnant symptomatic, or pregnant asymptomatic) the specimen should be evaluated. Once classified to an algorithm, specimens were then evaluated per the algorithm based on specimen type and the days collected post-symptom onset (see Figures 1 through 6). Key differences in algorithms include decreasing the collection window for rRT-PCR for direct detection of the virus, incorporating serological analyses for DENV to aid in discriminating infection type and timing, and expanding algorithm results to include presumptive infections (where PRNT

results are unavailable). Differences between the performance of the algorithms was assessed using the test of marginal homogeneity with a significance level of 0.05.

Results

A total of 1,522 cases, encompassing 3,321 individual specimens and 6,841 test results, were identified and included in this study. As expected, due to the focus of surveillance on pregnant women, most cases, 1,016 (66.8%), were identified in women (Table 1). Similarly, most identified cases (72.1%) were symptomatic. Of the 1,016 female cases with identified ZIKV, 37 (3.6%) were pregnant at the time of infection. The average age of symptomatic and asymptomatic patients was 40.7 and 31.1 years, respectively. Of note, no asymptomatic cases were identified in children aged 0 to 9. Most cases (n=1,201; 78.8%) indicated travel to at least one dengue-endemic country.

Table 1. Summary of case characteristics of cases with ZIKV infections in Florida, 2016-2017.

	Symptomatic	Asymptomatic	Unknown	Total
Gender				
Female	627 (57.1)	379 (92.2)	10 (76.9)	1,016 (66.8)
Male	471 (42.9)	32 (7.8)	3 (23.1)	506 (33.3)
Total	1,098 (72.1)	411 (27.0)	13 (0.9)	1,522 (100.0)
Diagnosis status				
Confirmed	964 (87.8)	56 (13.6)	4 (30.8)	1,024 (67.3)
Probable	134 (12.2)	355 (86.4)	9 (69.2)	498 (32.7)
Pregnancy status				
Pregnant	32 (2.9)	5 (1.2)	0 (0.0)	37 (2.4)
Not pregnant	595 (54.2)	374 (91.0)	10 (76.9)	979 (64.3)
Not applicable (male)	471 (42.9)	32 (7.8)	3 (23.1)	506 (33.3)
Age (years)				
Mean	40.7	31.1	32.4	38.1
Range	1-86	15-89	18-59	1-89
0-4	4 (0.4)	0 (0.0)	0 (0.0)	4 (0.3)

5-9	14 (1.3)	0 (0.0)	0 (0.0)	14 (0.9)
10-19	74 (6.7)	25 (6.1)	1 (7.7)	100 (6.6)
20-29	185 (16.9)	163 (39.7)	3 (23.1)	351 (23.1)
30-39	265 (24.1)	183 (44.5)	8 (61.5)	456 (30.0)
40-49	220 (20.0)	24 (5.8)	0 (0.0)	244 (16.0)
50-59	202 (18.4)	7 (1.7)	1 (7.7)	210 (13.8)
≥60	134 (12.2)	9 (2.2)	0 (0.0)	143 (9.4)
Travel to dengue-endemic countries*				
Endemic country	845 (77.0)	349 (84.9)	7 (53.9)	1,201 (78.9)
Sporadic or uncertain	19 (1.7)	7 (1.7)	0 (0.0)	26 (1.7)
Florida only†	228 (20.8)	51 (12.4)	6 (46.2)	285 (18.7)
Sexually acquired‡	6 (0.6)	0 (0.0)	0 (0.0)	6 (0.4)
Unknown	0 (0.0)	4 (1.0)	0 (0.0)	4 (0.3)

*CDC Yellow Book 2020 (19) used for determining DENV endemicity.
†Florida only cases had no recent travel.
‡Sexually acquired cases had no recent history of travel or known exposure to local transmission area, or had laboratory evidence of supporting sexual transmission.

Of the 3,321 specimens, 2,926 (88.1%) were tested by rRT-PCR for ZIKV; 1,423 (42.8%) by ZIKV IgM; 1,071 (32.2%) by DENV IgM; 699 (21.0%) by ZIKV PRNT; and 722 (21.7%) by DENV PRNT (Table 2). The most commonly tested specimen type was serum (1,954; 58.8%), followed by urine (1,259; 37.9%) and whole blood (108; 3.3%). Of those specimens tested by rRT-PCR, 45.3% (1,326) were reported as detected, indicating presence of the virus. ZIKV was detected most frequently in urine specimens (729 out of 1,259; 57.9%), followed by serum (568 out of 1,559; 36.4%) and whole blood (29 out 108; 26.9%). Notably, only 4.1% (24) of asymptomatic cases tested positive for ZIKV by rRT-PCR as compared to 56.1% (1,300) of symptomatic cases. Conversely, 72.6% (365) of asymptomatic cases were positive for ZIKV IgM as compared to 67.6% (612) of symptomatic cases. Of the 1,112 sera that were tested by both ZIKV rRT-PCR and IgM, only 10.7% (119) were positive in both tests.

Table 2. Summary of laboratory results for 3,321 specimens from 1,522 cases with ZIKV infections in Florida, 2016-2017.

	Symptomatic	Asymptomatic	Unknown	Total
ZIKV RT-PCR, n (%)				
Detected	1,300 (56.1)	24 (4.1)	2 (14.3)	1,326 (45.3)
Equivocal	78 (3.4)	2 (0.3)	0 (0.0)	80 (2.7)
Not detected	941 (40.6)	567 (95.6)	12 (85.7)	1,520 (2.0)
Total	2,319 (79.3)	593 (20.3)	14 (0.5)	2,926(100.0)
ZIKV IgM, n (%)				
Positive	612 (67.6)	365 (72.6)	11 (78.6)	988 (69.4)
Equivocal	38 (4.2)	74 (14.7)	1 (7.1)	113 (7.9)
Negative	245 (27.0)	39 (7.8)	2 (14.3)	286 (20.1)
Inconclusive	1 (0.1)	1 (0.2)	0 (0.0)	2 (0.1)
Indeterminate	10 (1.1)	24 (4.8)	0 (0.0)	34 (2.4)
Total	906 (63.7)	503 (35.4)	14 (1.0)	1,423 (100.0)
DENV IgM, n (%)				
Positive	109 (15.1)	32 (9.4)	2 (25.0)	143 (13.4)
Equivocal	9 (1.2)	5 (1.5)	0 (0.0)	14 (1.3)
Negative	604 (83.5)	300 (88.2)	6 (75.0)	910 (85.0)
Inconclusive	0 (0.0)	2 (0.6)	0 (0.0)	2 (0.2)
Indeterminate	1 (0.1)	1 (0.3)	0 (0.0)	2 (0.2)
Total	723 (67.5)	340 (31.8)	8 (0.8)	1,071 (100.0)
ZIKV PRNT, n (%)				
Positive	261 (98.5)	423 (99.8)	10 (100.0)	694 (99.3)
Negative	4 (1.5)	1 (0.2)	0 (0.0)	5 (0.7)
Total	265 (37.9)	424 (60.7)	10 (1.4)	699 (100.0)
DENV PRNT, n (%)				
Positive	155 (56.4)	395 (90.6)	9 (81.8)	559 (77.4)
Negative	120 (43.6)	41 (9.4)	2 (18.2)	163 (22.6)
Total	275 (38.1)	436 (60.4)	11 (1.5)	722 (100.0)

1,423 specimens (72.8% of serum specimens collected) were tested for IgM against ZIKV; 988 (69.4%) were positive. 1,071 specimens (54.7% of serum specimens collected) were tested for IgM against DENV. DENV IgM was positive in 13.4% (143) of these specimens. Of those specimens that were tested by both DENV IgM and ZIKV

IgM (n=982), 12.9% (127) of those that tested positive for IgM against ZIKV also tested positive for DENV IgM. In specimens that tested positive by rRT-PCR for ZIKV and were tested for IgM against DENV, 9.0% (19) were positive for both.

Six hundred ninety-nine and 722 sera were tested for ZIKV and DENV by PRNT, respectively. Of these, 99.3% (694) were positive for ZIKV, and 77.4% (559) were positive for DENV. Both test results were available for 691 sera, and 420 of these specimens were associated with asymptomatic cases, only 4 of which were from pregnant cases. Of the 691 sera tested by both ZIKV and DENV PRNT, 531 (76.8%) were positive for both ZIKV and DENV, 155 (22.4%) were positive for ZIKV alone, and 5 (0.7%) were negative for both viruses.

Using the CDC 2017 testing guidelines, 1,105 cases would have been eligible for testing: 1,066 cases would have been tested using the symptomatic non-pregnant algorithm, 32 cases with the symptomatic pregnant algorithm, and 5 with the asymptomatic pregnant algorithm. Four hundred nineteen cases were identified by the Florida DOH that would not have been tested according to these recommendations (Table 3). Figures 1-6 depict the number of specimens and cases in each of the 3 algorithms for 2017 and 3 algorithms for 2019. After accounting for duplicate cases, the 2017 algorithm identified 820 cases (74.3%) that would have been adjudicated as acute infections with ZIKV, 75 (6.8%) as infections with ZIKV with undetermined timing, 102 (9.2%) as flavivirus infections with undetermined timing, 39 (3.5%) as no evidence of ZIKV infection, 32 (2.9%) with no algorithm result, and 35 (3.2%) that would have been excluded due to collection timing. The 2019 algorithm identified 761 (69.0%) acute infections, 140 (12.7%) recent or presumptive ZIKV infections, 112 (10.2%) recent or

presumptive flavivirus infections, 1 (0.1%) presumptive DENV infection, 19 (1.7%) with no evidence of ZIKV or DENV infection, 26 (2.4%) with no algorithm result, and 44 (4.0%) that were excluded for testing due to collection timing.

Table 3. Theoretical comparison of 2017 and 2019 Centers for Disease Control and Prevention Algorithms' performance---Results that would have been seen had each of the algorithms been used.

	2017 Algorithms				2019 Algorithms			
	NPS*	PS	PA	Total	NPS	PS	PA	Total
Total specimens in category	2,288	179	34	2,501	2,288	179	34	2,501
Patients represented in category	1,066	32	5	1,103	1,066	32	5	1,103
Specimens collected ≤13 or 7 days post-symptom onset‡	1,817	54	-	1,871	1,581	41	-	1,622
Specimens collected >13 or 7 days post-symptom onset‡	254	125	-	379	256	107	-	363
Specimens tested by ZIKV NAAT	1,753	171	30	1,954	1,531	141	30	1,702
Positive (%)	1,153 (65.8)	74 (43.3)	6 (20.0)	1,233 (63.1)	1,064 (69.5)	70 (49.7)	6 (20.0)	1,140 (67.0)
Negative or equivocal (%)	597 (34.1)	97 (56.7)	24 (80.0)	718 (36.7)	467 (30.5)	71 (50.4)	24 (80.0)	562 (33.0)
Specimens tested by ZIKV and/or DENV IgM	591	95	-	686	596	78	-	674
Non-negative (%)	502 (84.9)	84 (88.4)	-	586 (85.4)	565 (94.8)	69 (88.5)	-	634 (94.1)
Negative (%)	89 (15.1)	11 (11.6)	-	100 (14.6)	31 (5.2)	9 (11.5)	-	40 (5.9)

Tested by ZIKV and DENV PRNT ZIKV ≥10 DENV <10	235	13	-	248	207	11	-	218
ZIKV ≥10 DENV ≥10	107 (45.5)	4 (30.8)	-	111 (44.8)	105 (50.7)	3 (27.3)	-	108 (49.5)
ZIKV <10 (DENV <10)	126 (53.6)	9 (69.2)	-	135 (54.4)	99 (47.8)	8 (72.7)	-	107 (49.1)
	2 (0.9)	0 (0.0)	-	2 (0.8)	3 (1.4)	0 (0.0)	-	3 (1.4)
Case determination								
Acute ZIKV infection	783 (73.5)	32 (100.0)	5 (100.0)	820 (74.3)	726 (68.1)	30 (93.8)	5 (100.0)	761 (69.0)
Recent or presumptive ZIKV infection	75 (7.0)	0 (0.0)	0 (0.0)	75 (6.8)	140 (13.1)	0 (0.0)	0 (0.0)	140 (12.7)
Recent or presumptive flavivirus infection	102 (9.6)	0 (0.0)	0 (0.0)	102 (9.2)	111 (10.4)	1 (3.1)	0 (0.0)	112 (10.2)
Recent or presumptive DENV infection	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.1)	0 (0.0)	0 (0.0)	1 (0.1)
No evidence of ZIKV or DENV infection	39 (3.7)	0 (0.0)	0 (0.0)	39 (3.5)	19 (1.8)	0 (0.0)	0 (0.0)	19 (1.7)
No algorithm result	32 (3.0)	0 (0.0)	0 (0.0)	32 (2.9)	26 (2.4)	0 (0.0)	0 (0.0)	26 (2.4)
Excluded from testing	35 (3.3)	0 (0.0)	0 (0.0)	35 (3.2)	43 (4.0)	1 (3.1)	0 (0.0)	44 (4.0)

*NPS: non-pregnant symptomatic cases; PS: pregnant symptomatic cases; PA: pregnant asymptomatic cases

† p-value from two sample proportions test (two-tailed) between algorithm totals; results significant at $p<0.05$.

‡Specimens collected ≤13 and 7 days post-symptom onset for the 2017 and 2019 algorithms, respectively.

Figure 1. 2017 CDC recommended testing algorithm for non-pregnant, asymptomatic cases.

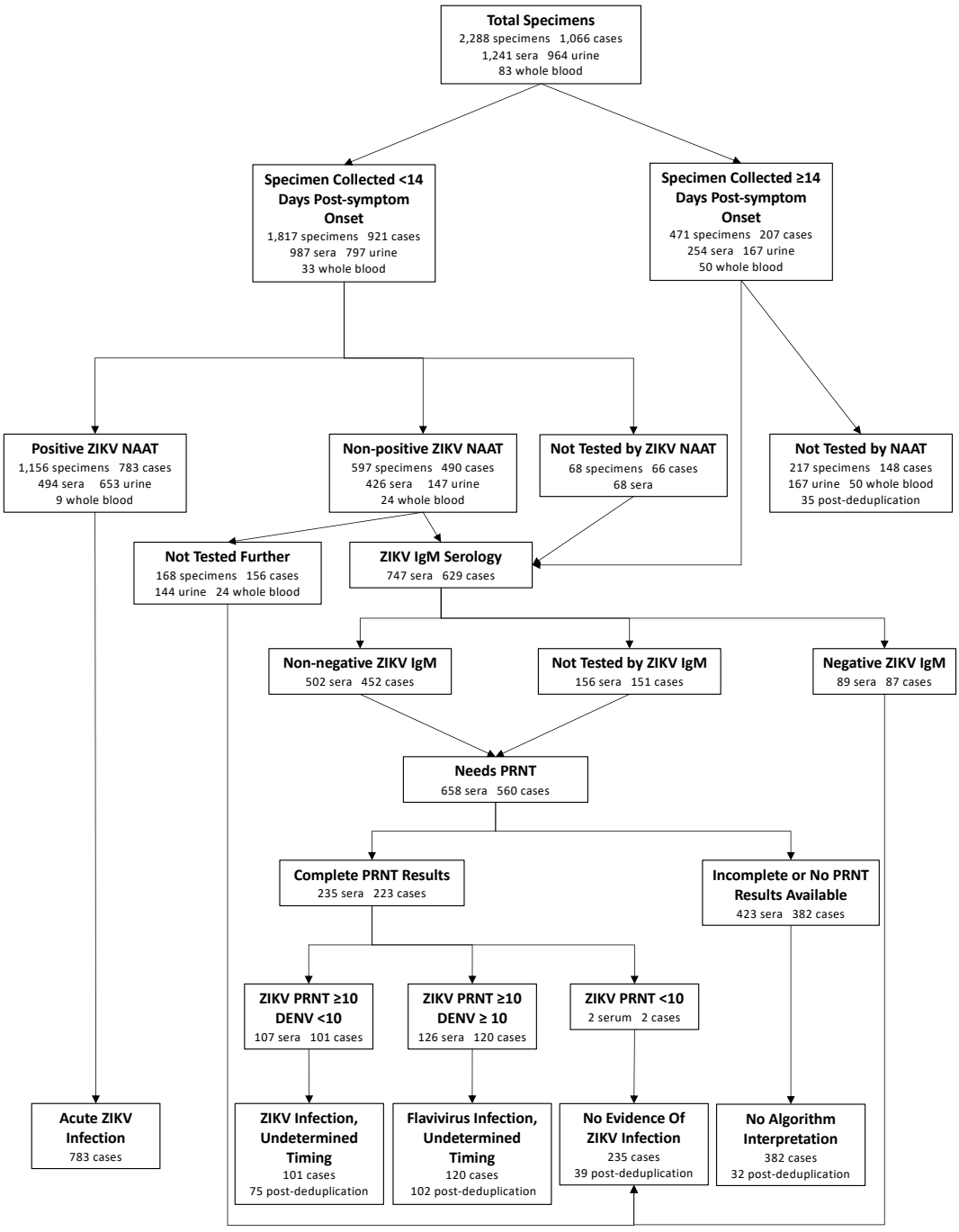


Figure 2. 2017 CDC recommended testing algorithm for pregnant, symptomatic cases.

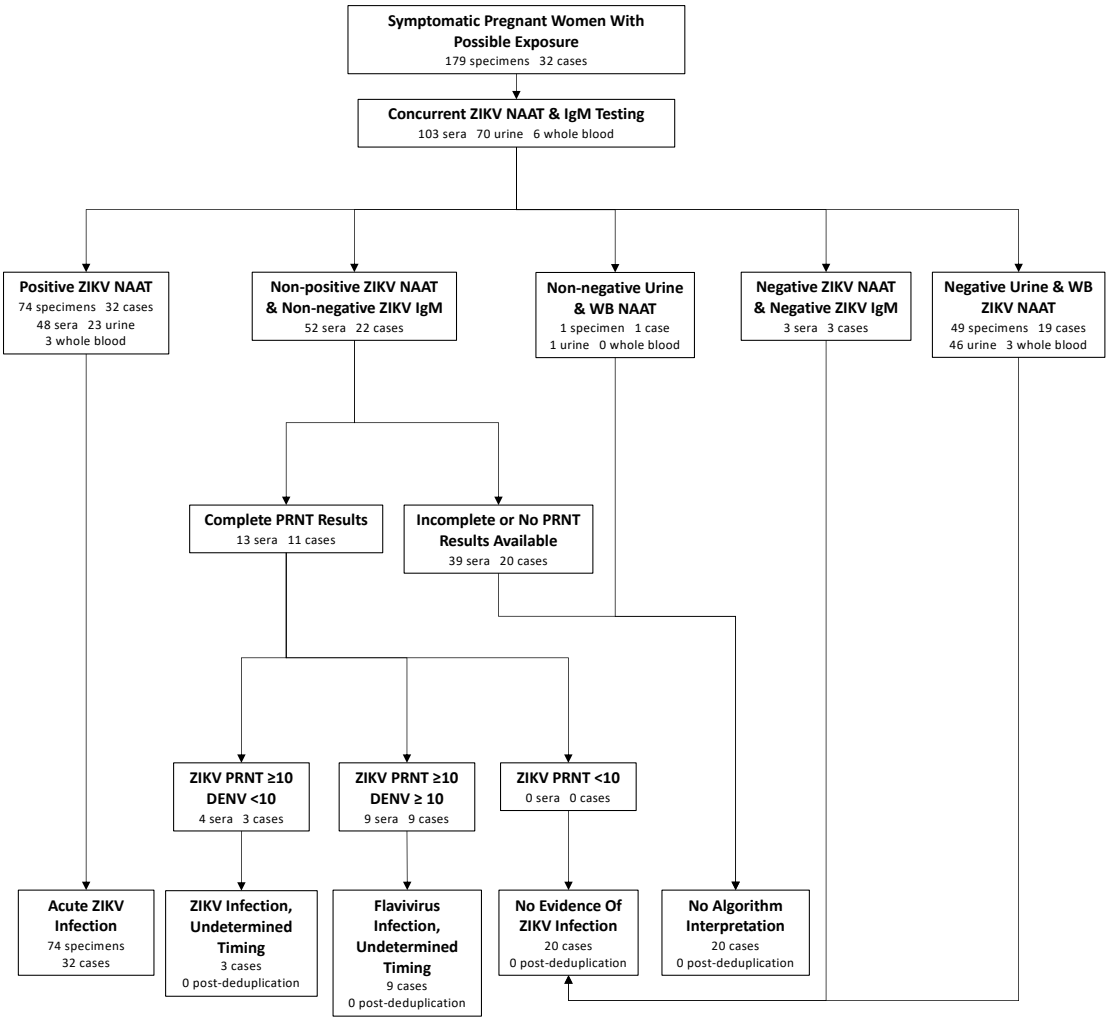


Figure 3. 2017 CDC recommended testing algorithm for pregnant, asymptomatic cases.

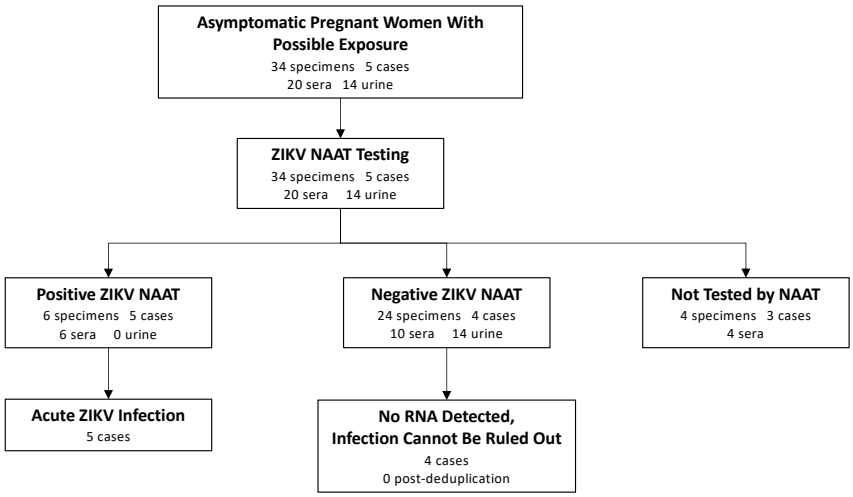


Figure 4. 2019 CDC recommended testing algorithm for non-pregnant, asymptomatic cases.

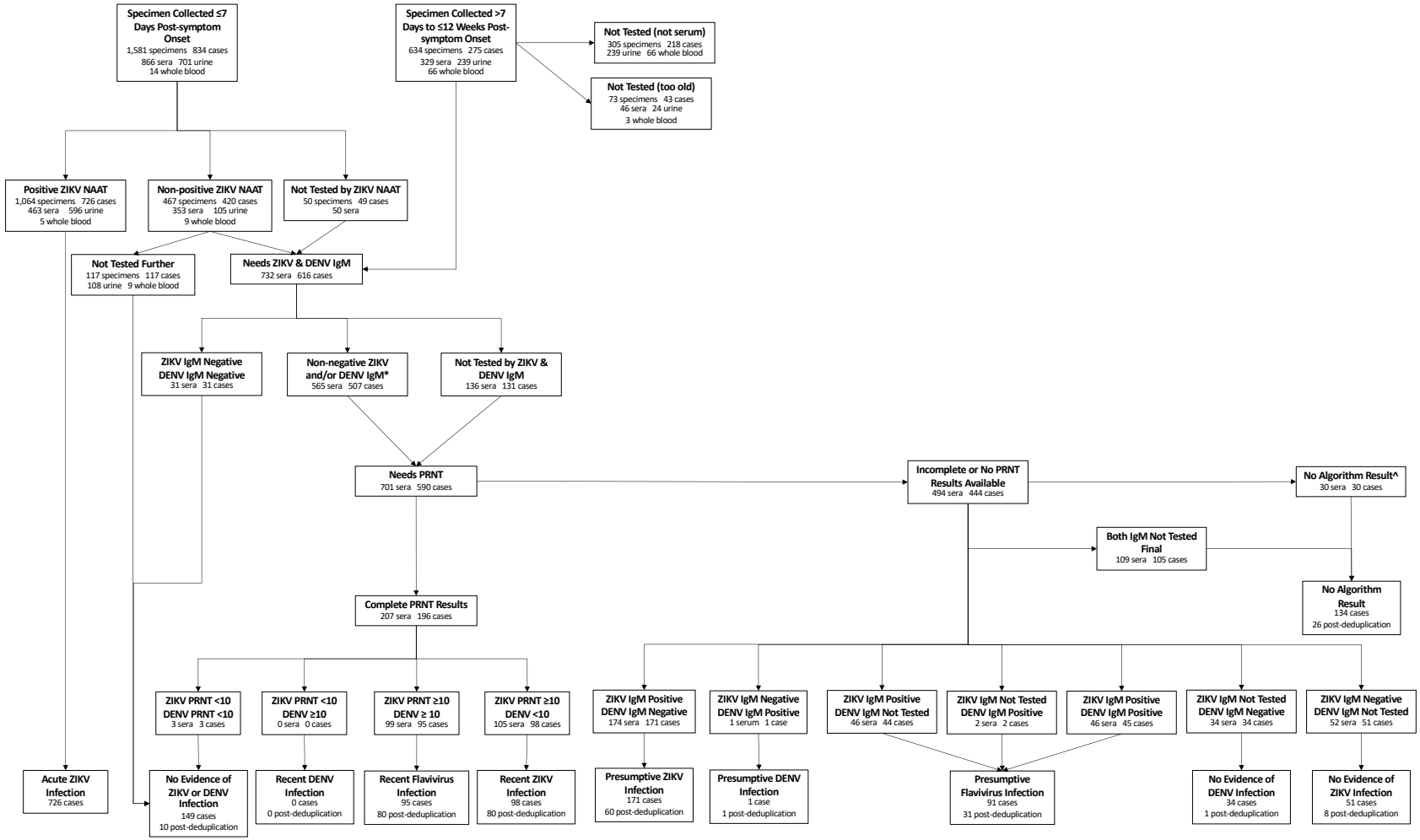


Figure 5. 2019 CDC recommended testing algorithm for pregnant, symptomatic cases.

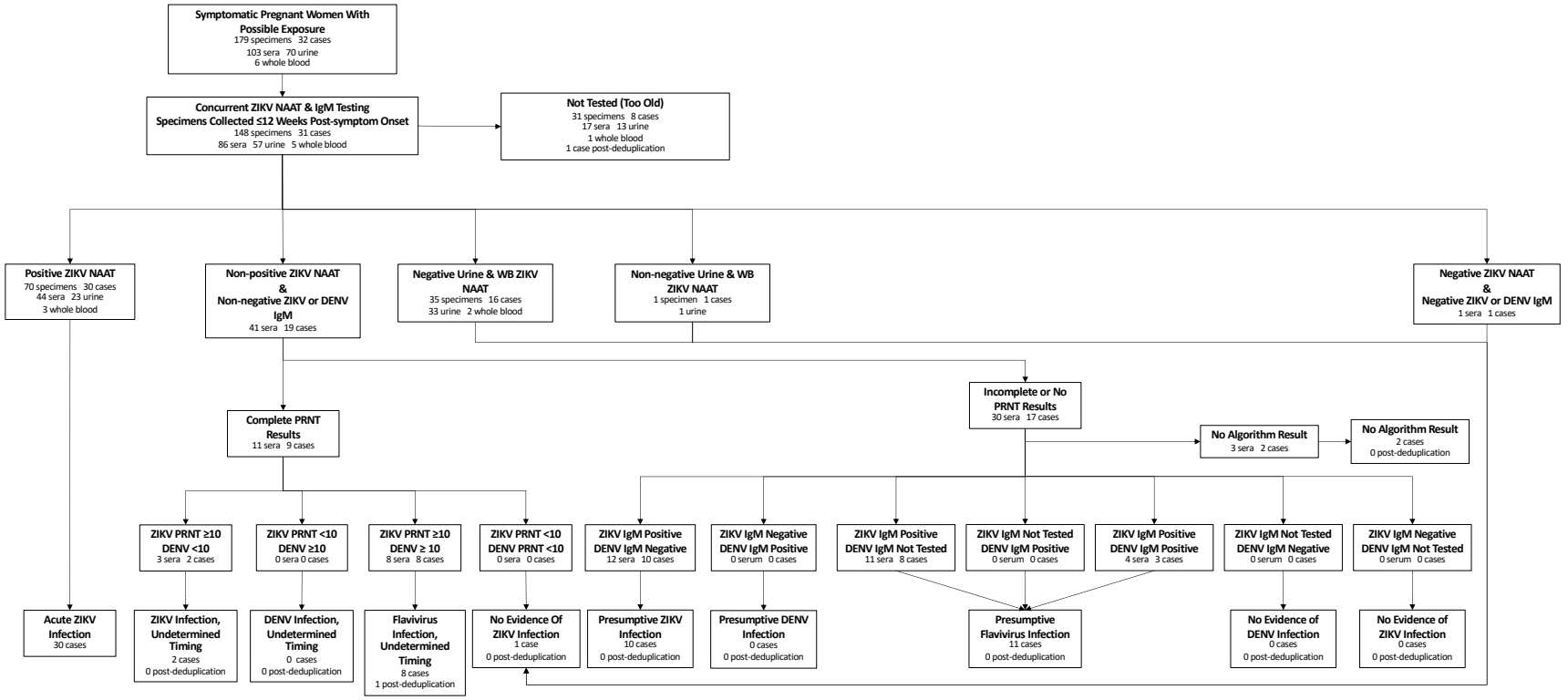
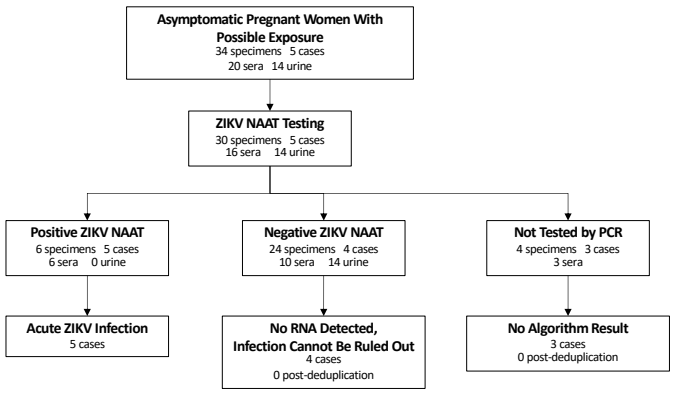


Figure 6. 2019 CDC recommended testing algorithm for pregnant, asymptomatic cases.



When analyzed using the 2017 algorithm, only 44.8% (111) of those specimens tested against both ZIKV and DENV for PRNT were positive against only ZIKV; 49.5% (108) of those analyzed with the 2019 algorithm were positive against only ZIKV. 135 (54.4%) and 107 (49.1%) of specimens were positive against both ZIKV and DENV using the 2017 and 2019 algorithms, respectively. Interestingly, only 30.8% (4) and 27.3% (3) of specimens from pregnant, symptomatic women were positive against only ZIKV with the 2017 and 2019 algorithms, respectively.

Compared with the 2019 testing algorithm, the 2017 testing algorithm resulted in definitively identifying 59 more cases as acute infections. In addition to identifying fewer acute infections, the 2019 testing algorithm identified almost twice as many recent or presumptive cases of ZIKV infections than the earlier algorithm. Conversely, the 2019 algorithm categorized approximately half as many cases as having no evidence of ZIKV or DENV infection. Overall, there was a significant difference between the two algorithms ($x^2 = 81.2$, df = 6, $p < 0.001$).

Discussion

Retrospective analysis of the 2017 and 2019 testing algorithms demonstrates the challenging task of diagnosing ZIKV infections. The CDC strategy relies upon separating potential cases by presentation of symptoms and further focusing testing efforts on pregnant women due to the increased risks associated with ZIKV. This strategy focuses resources on the population most severely affected by ZIKV and attempts to limit false-positive results that could result in anxiety in pregnant mothers and unnecessary abortions. However, a major limitation to this approach is that there is no allowance for testing of non-pregnant, asymptomatic patients. Between 2016 and 2017, the Florida DOH identified 419 confirmed and probable cases of ZIKV infection in non-pregnant, asymptomatic patients. This accounted for 27.5% of all cases in the dataset. With both the 2017 and 2019 testing algorithms, these patients would not have been tested. This group must be accounted for when investigating potential cases or outbreaks and setting up surveillance systems as asymptomatic cases are thought to account for approximately 80% of all cases (4) and can serve as a source of infection (20).

Evidence suggests that the viral load observed in patients infected with ZIKV is generally low (6, 9, 10), limiting the utility of rRT-PCR. The 2019 algorithm limits the specimen collection window to ≤7 days compared to ≤13 days in the 2017 algorithm. Of the 1,326 PCR-positive specimens with associated collection dates, 1,097 (82.7%) specimens were collected ≤7 days. Extending the collection window to ≤13 days increases the number of positive results to 1,202 (90.6%) specimens. If the collection window was expanded to ≤28 days, 95.2% (1,262) of the specimens that tested positive by rRT-PCR would be included in the algorithm. Part of the increase in recent or

presumptive ZIKV infections observed in the 2019 algorithm as compared to the 2017 algorithm can be attributed to the shortening of the specimen collection window for rRT-PCR testing. Of these additional 68 cases, 23.5% (16 cases) would have been adjudicated as acute cases with a collection window ≤13 days; this increases to 30.9% (21 cases) with a specimen collection window of ≤28 days. A larger testing window, for ZIKV may be indicated as detection of viral RNA allows investigators to unequivocally determine the timing of infection as compared to serological testing. Because ZIKV RNA was detected in far fewer asymptomatic cases (4.1%), testing of non-pregnant, asymptomatic patients could potentially be limited to further optimize future algorithms while expanding specimen collection windows. However, even a small increase in the number of cases adjudicated by rRT-PCR could potentially save time and resources in performing extraneous serological testing due to the high specificity of the assay. Further, rRT-PCR generally has a much quicker turnaround time and is much more readily available in laboratories as compared to IgM and PRNT testing. Real-time RT-PCR capacity is also much easier to quickly expand in the event of an outbreak (21, 22).

Of specimens tested for IgM antibodies against both ZIKV and DENV, 12.9% were positive for both, demonstrating the potential for cross-reactivity. This concurs with other studies that have assessed the cross-reactivity of the two viruses (7). In a cohort of 61 patients diagnosed with acute ZIKV infection from Brazil, Felix and colleagues evaluated the cross-reactivity with several DENV IgM assays, finding up to 16.4% and 37.7% cross-reactivity, depending on the assay and date of collection (23). This level of cross-reactivity and the potential for false-positive results make relying upon ZIKV IgM assays challenging, especially when patient treatment and other decision-making is

dictated by the outcome. This is further complicated in locations where DENV is prevalent or populations with increased prior exposure to DENV.

The performance and resulting utility of the PRNT assay for the determination of disease etiology is limited. About half of the cases with both ZIKV and DENV PRNT results were able to distinguish ZIKV as the etiology according to the 2017 and 2019 algorithms, 44.8% and 49.5% respectively. The percentage was even lower in pregnant cases, although the sample size for this group is relatively small. The inability of the PRNT assay to identify the specific etiology for such a large percentage of cases may be, in part, due to the exposure of patients to other flaviviruses, such as DENV, through travel. The large number of foreign-born residents in Florida, and in Miami-Dade County in particular, may also influence the performance of this assay (8). With the amount of effort, time, and resources that goes into performing these tests, the resulting outcome needs to be considered. Additionally, the PRNT assay is a very specialized test, relying on time-consuming cell culture methods, and is not readily available in most laboratories. In Florida, the PRNT assay is only performed in one laboratory in the state, BPHL-Tampa, creating potential delays in testing due to shipping requirements. As in other locations, the inclusion of the PRNT assay must be considered within the context of geography and the outbreak itself (11, 12, 24).

The CDC recently revised its testing guidance for ZIKV given the decreased risk of transmission world-wide and the prolonged persistence of IgM antibodies to ZIKV (25, 26). This update only recommends serologic testing in cases consistent with congenital ZIKV infection and residence or travel to an area at risk of ZIKV; ZIKV IgM

testing is not recommended for symptomatic or asymptomatic pregnant patients. This will place more reliance on rRT-PCR testing to identify outbreaks of ZIKV in the future.

These analyses are subject to several limitations. This study utilizes laboratory and epidemiological data collected during the ZIKV outbreak from 2016 to 2017 to retrospectively test these algorithms. This approach treats specimens from the same patient collected on different dates and analyzes them independently. In a practical setting, multiple laboratory results from multiple specimens would be analyzed together, along with other available data, to determine the etiology and nature of infection. De-duplicating cases hierarchically attempts to account for this. However, this approach also clearly demonstrates the variability of results across time and the difficulty of diagnosing ZIKV infections with currently available laboratory tests. Using only confirmed and probable cases also limits the utility of this analysis to evaluating the efficacy in terms of presumably true cases. To comprehensively evaluate testing guidance and assays, the inclusion of non-case data is also important. This additional data would allow for evaluation of non-ZIKV infections in the testing algorithms, such as those caused by DENV.

Epidemiologists and health care providers need to be cognizant of the limitations of testing for ZIKV. Current guidance for testing is limited in that it potentially does not account for approximately 80% of cases as testing of asymptomatic patients is limited to pregnant patients. This guidance focuses on patient diagnosis for those most at risk and does not account for critical public health surveillance activities. Public health agencies will need to adapt these recommendations in order to effectively detect and identify future transmission of ZIKV. In the context of an outbreak, asymptomatic people must be

included on the basis of epidemiological factors in an attempt to balance disease detection and laboratory resources. Expansion of the specimen collection window for rRT-PCR testing, especially in response to an outbreak, can potentially maximize the availability of laboratory resources by limiting the need for serological testing. This can also include use of the PRNT assay, which, in some populations, may be of limited utility.

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Chapter 6

Conclusion and Recommendations

Conclusion

The unprecedented worldwide spread of ZIKV offered a unique opportunity to study a once rare infection. Because of the significant increase in infections, complications with fetal development and an increased risk of neurologic complications were identified as being associated with ZIKV infections. The surge in cases also brought the development and implementation of new diagnostic assays, providing a wealth of both clinical and laboratory data. The studies presented herein sought to capitalize on this increase in evidence to investigate the natural history of the virus.

The first study, a systematic review of published cases, estimated the median incubation period of ZIKV disease to be 6.5 days from exposure. Abstracted data were also used to calculate median viral RNA clearance time from symptom onset from various clinical specimens. Vaginal and serum specimens were observed to have the quickest time to viral clearance, 9.9 and 10.8 days, respectively; whereas, semen and whole blood specimens had the longest time to viral clearance, 47.7 and 49.2 days, respectively. Saliva and urine had similar times to viral clearance, 18.9 and 20.5 days, respectively. These estimates can be used to adapt testing methodologies and algorithms to optimize the detection of infections with ZIKV. This can be especially important given the challenges of identifying acute infections using serological methods, such as IgM or PRNT.

As an extension of viral clearance time, available viral isolation data were examined as a proxy for infectivity. Due to both the tremendous variability in viral

isolation methods and the inconsistency in reporting these methods, it was difficult to make direct comparisons and analyze these variables in aggregate. Overall, only 13.9% of viral isolation attempts resulted in recovery of the virus. Semen, serum, and urine all exhibited similar recovery rates. However, virus was not recovered from whole blood in any of the 33 attempts, indicating it is a poor specimen choice for viral isolation attempts. This indicates that, although ZIKV can be detected longest in whole blood specimens by rRT-PCR, these specimens may not actually be infectious for the entire duration that in which ZIKV can be detected by rRT-PCR.

The second study built upon this systematic review by utilizing data collected by the Florida DOH on confirmed and probable symptomatic cases between 2016 and 2017. Of the 2,044 specimens included in the study, ZIKV was detected most often in urine specimens, and, when collected alongside serum specimens, were detected in 75.4% of specimens as compared to 44.4% of serum specimens. Within the first four weeks following symptom onset, ZIKV RNA was detected at higher rates in urine specimens as compared to serum specimens. Urine also had the lowest median C_t value, indicating a significantly higher viral load than whole blood or serum. These findings indicate that urine may be the ideal specimen for detecting ZIKV RNA by rRT-PCR. However, almost a third of positive whole blood specimens were detected ≥28 days post-symptom onset, indicating the potential utility of using whole blood to diagnose older infections.

The final study examined case data from the ZIKV outbreak in Florida from 2016 to 2017 in the context of diagnostic algorithms. Epidemiological and laboratory data were utilized to retrospectively assess the efficacy of the CDC's recommend algorithms for diagnosing Zika virus disease. The transition to a shorter recommended collection time

for molecular testing led to fewer determinations of acute ZIKV infection and a corresponding increase in the identification of recent or presumptive cases of ZIKV infection. Both algorithms exclude testing of specimens from asymptomatic people who are not pregnant. In the experience of Florida, strict application of this recommendation would have resulted in the potential failure to detect 419 cases.

Within this dataset, 12.9% of specimens tested for IgM antibodies against both ZIKV and DENV were positive for both. Of the 691 specimens tested by both ZIKV and DENV PRNT, 531 (76.8%) were positive for both viruses. Nine percent of specimens that were positive for ZIKV by rRT-PCR and tested for IgM antibodies against DENV were also positive by DENV IgM. This adds to the body of evidence that serological methods for flaviviruses are non-specific, causing difficulties in interpreting their results. Increased reliance on molecular detection and a larger specimen collection window could potentially alleviate the reliance upon serological methods while still identifying the majority of cases.

Recommendations

These findings underscore the importance that laboratory testing plays when responding to public health emergencies. Especially with an infection where a large proportion of cases are asymptomatic or symptoms are mild or non-pathognomonic, appropriate utilization and interpretation of laboratory testing is critical. When resources are limited, these factors become even more important. With regard to ZIKV, these studies add to the mounting evidence that urine may be the optimal specimen to utilize as a diagnostic specimen. Given the higher viral load, the ability to detect ZIKV RNA

within the first three weeks of infection with urine, and the relative ease of specimen collection, prioritizing urine collection may aid in identifying more acute cases. Additionally, urine is a relatively simple and non-invasive specimen to collect, making it ideal for surveillance studies.

Utilization of urine alone, however, may not be the optimum strategy. Given the lengthened time to viral clearance for whole blood specimens, there is a place for whole blood in the diagnostic algorithm as well. Whole blood could potentially be used in cases where infection with ZIKV is highly suspected and other etiologies are ruled out. These studies suggest that ZIKV RNA could be detected in whole blood for upwards of two months following symptom onset. If serological testing is inconclusive in such cases, testing whole blood can be considered.

Further review of the window for specimen collection for routine molecular testing needs to be reexamined. Current CDC guidelines recommend limiting molecular testing to specimens collected within 7 days from symptom onset. These studies demonstrate that ZIKV RNA can be regularly detected in urine specimens for at least two weeks, if not three. As mentioned previously, further analyses must be conducted to determine if the additional testing volume would be offset by the ability to identify these additional cases. Given the challenges with serological testing and widespread ability to implement molecular testing, it appears this additional testing may be worthwhile.

Lastly, current testing algorithms do not account for asymptomatic cases in non-pregnant individuals. Given that up to 80% of all infections with ZIKV are asymptomatic and that most infections are mild, consideration for testing asymptomatic patients must be given. Strategies must be devised to account for this population without overloading the

laboratory system. Pooling specimens from asymptomatic patients may be an effective approach that can strike a balance with the volume of testing and the benefit for public health surveillance.

The unprecedented emergence of ZIKV illuminated many of the deficiencies within our global health system. The pandemic, however, taught the lesson of flexibility. With the increased number of cases, came an increase in our knowledge of the virus and its effects on its host. Leveraging this lesson and newfound knowledge will be critical in responding and controlling the next outbreak of ZIKV and other emerging illnesses.

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