

Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

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SUPPLEMENT

Zika virus persistence in body fluids

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A. Methods

Specimen collection

Participants chose to attend the visit at the study site or have a home visit to complete study procedures. Serum was collected via venipuncture. Saliva, urine, and semen (adults only) were self-collected in sterile containers. Adult women self-collected vaginal swabs. Specimens were collected at the initial visit, every week for four weeks, and bimonthly for patients in which ZIKV was not detected by RT-PCR in any specimen at week 4. For patients in which ZIKV was detected by RT-PCR in any specimen at week 4, specimen collection continued biweekly until all specimens became negative, and specimens were collected bimonthly thereafter for up to 6 months.

Laboratory methods

Specimens were refrigerated at 4°C upon collection and transported within 24 hours to the Centers for Disease Control and Prevention (CDC) Dengue Branch laboratory in San Juan, Puerto Rico. Semen and saliva specimens were frozen at -70°C upon arrival at the laboratory and the other specimen types were kept refrigerated at 4°C until tested. Vaginal swabs and saliva (the latter only when specimen was not sufficient) were diluted in viral transport medium. For each specimen, 200 µL were tested with the CDC Trioplex Real Time RT-PCR assay for the detection of dengue, chikungunya, and Zika viral RNA. The Trioplex assay includes an internal control for the detection of human RNase P (RP) transcripts in clinical specimens which functions as an indicator of the RNA extraction and RT-PCR process. The Trioplex RT-PCR assay was evaluated for semen. Specimens were considered positive if target amplification was detected within 38 threshold amplification cycles (CT). The RNA extraction and real-time RT-PCR process were considered valid if the RP reaction was positive. Intermittent RNA detection was defined as detection of viral RNA followed by lack of detection followed by detection, regardless of

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the time interval between specimen collections. All serum specimens were also tested by anti-ZIKV IgM antibody capture enzyme-linked immunosorbent assay (ZIKV MAC-ELISA) following protocol in accordance to the CDC Zika MAC-ELISA Instructions for use document.

B. Validation of the Trioplex RT-PCR for Zika virus RNA detection in semen

Background

The performance of the Trioplex in saliva and vaginal secretions has not been established; saliva and vaginal specimens were processed with the same protocol as serum and urine specimens. We report on the validation of the Centers for Disease Control and Prevention (CDC) Trioplex Real Time reverse transcriptase-polymerase chain reaction (RT-PCR) for detection of Zika virus (ZIKV) RNA in semen specimens from healthy volunteers.

Methods

Semen was collected from two healthy volunteers attending the GENES Fertility Clinic in San Juan, Puerto Rico. Two frozen human semen collections were thawed at room temperature and pooled. ZIKV French Polynesia 2013 strain previously grown by tissue culture at the CDC Dengue Branch laboratory was diluted 1:100 in Dulbecco's Modified Eagle Medium and used to spike the pooled semen. Commercial normal human serum (Corning) was used as control specimen and spiked with the same virus suspension. Four 1:10 serial dilutions were prepared for each specimen type corresponding to 1000x, 100x, and 10x above limit of detection and at the limit of detection. Each dilution was aliquoted 8 times into individual microcentrifuge tubes, mixed with 0.3mL of MagNA Pure LC lysis buffer (Roche, Indianapolis, IN) by vortex and incubated at room temperature for 15 minutes. ZIKV RNA from each aliquoted dilution was extracted in the MagNA Pure LC 2.0 (Roche) automated RNA extraction platform using the small-volume external lysis protocol. Real Time RT-PCR was conducted using the SuperScript III Platinum One-Step qRT-PCR mastermix (ThermoFisher, Waltham, MA) following the CDC Trioplex Real Time RT-PCR Assay Instructions for Use (30) including RNaseP (RP) internal control reactions on the ABI7500 Fast Dx instrument (ThermoFisher).

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Specimens are considered positive if target amplification is detected within 38 amplification cycles (CT).

The RNA extraction and real time RT-PCR process is considered valid if the RP reaction is positive. Here we present CT values and ZIKV genome copies equivalents per milliliter (GCE/mL) for serum and semen for each of the 8 replicas per dilution. Average CT, standard deviation, and average GCE/mL were estimated for each sample.

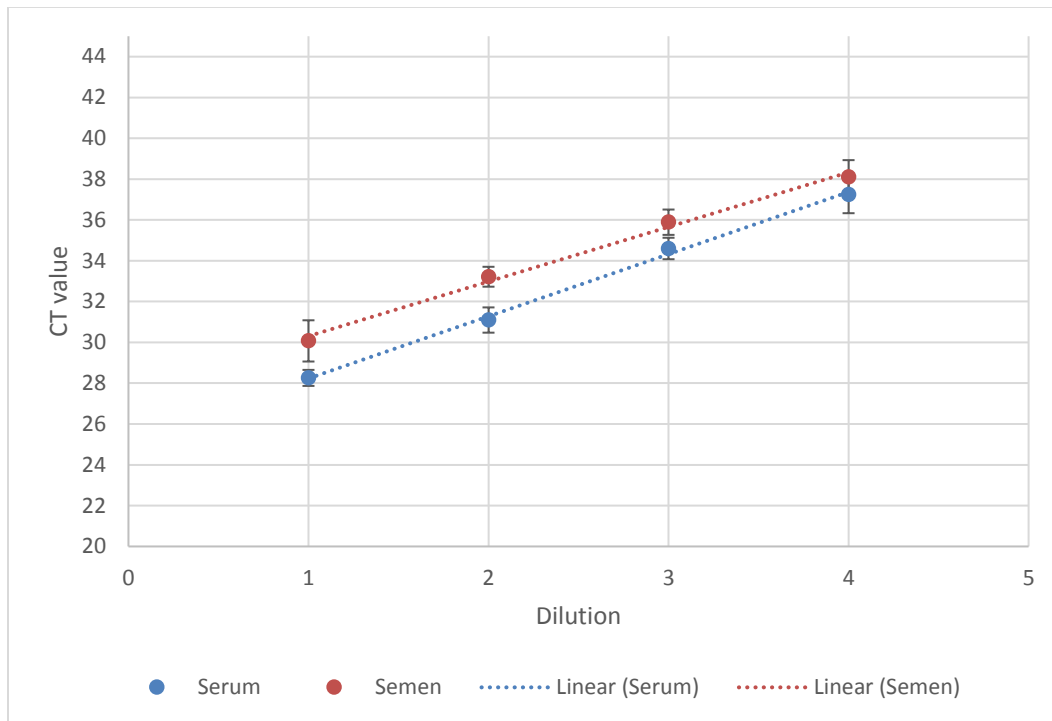
Results

The CDC Triplex Real Time RT-PCR Assay clearly detected and amplified ZIKV in semen comparable to serum up to the same limit of detection as described in the CDC Triplex Real Time RT-PCR Assay Instructions for Use approved under emergency use authorization issued by the US Food and Drug Administration. CT values were similar for each of the replicas at each dilution, suggesting that the assay is reproducible (Table B1). CT readings for serum and semen at each dilution, show that the assay has similar sensitivity and level of detection in the two specimens (Figure B1). RP internal control reactions tested positive on all samples indicating that all assay conditions were successful at target RNA extraction and nucleic acid amplification.

Conclusions

Our internal evaluation of the CDC Triplex Real Time RT-PCR Assay in semen showed that the assay is capable of processing semen samples, reproducible, and has similar sensitivity in semen and serum.

Figure B1. Triplex RT-PCR mean ZIKV CT values and standard deviation among 8 replicas of serum and semen specimens



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Table B1. Zika virus Triplex RT-PCR validation results in semen against serum

	ZIKV Diluted in Serum									ZIKV Diluted in Semen							
	1		2		3		4			1		2		3		4	
Replica #	Zika CT	RP CT	Zika CT	RP CT	Zika CT	RP CT	Zika CT	RP CT	Replica #	Zika CT	RP CT	Zika CT	RP CT	Zika CT	RP CT	Zika CT	RP CT
1	28.44	33.59	31.86	33.45	35.43	32	37.99	33.78	1	29.75	22.65	33.07	22.48	35.11	23.14	37.14	22.81
2	28.04	38.93	31.56	35.1	34.83	34.85	36.30	37.39	2	28.76	23.26	32.62	22.99	35.65	23.22	37.40	22.81
3	27.71	33.94	31.11	33.13	34.48	35.99	38.03	31.78	3	32.23	22.74	33.77	22.71	35.78	22.7	37.50	22.37
4	27.84	32.78	30.08	34.66	34.74	31.67	37.62	32.67	4	29.96	22.93	33.73	23.35	36.41	23.19	38.95	23.46
5	28.13	32.84	31.44	31.46	34.13	38.75	35.90	32.42	5	29.44	23.09	33.61	22.78	36.18	22.95	39.14	22.67
6	28.74	32.74	30.27	34.17	34.10	34.09	37.14	33.54	6	30.03	23.08	32.61	23.34	36.93	23.42	37.89	22.84
7	28.41	32.06	31.32	34.44	35.13	34.04	38.41	33.89	7	29.87	22.8	33.46	22.62	35.13	22.85	37.65	22.28
8	28.76	36.91	31.11	32.81	33.96	33.11	36.56	34.04	8	30.53	23.3	32.85	22.98	35.91	23.34	39.11	23.22
Mean CT	28.26	32.92	31.09	33.73	34.60	31.55	37.24	34.23	Mean CT	30.07	22.98	33.22	22.91	35.89	23.10	38.10	22.81
Std. dev.	0.39	2.41	0.62	1.18	0.53	2.29	0.91	1.70	Std. dev.	1.01	0.24	0.49	0.32	0.62	0.25	0.83	0.39
GCE/mL	3.17E+06		4.28E+05		3.24E+04		5.40E+03		GCE/mL	9.87E+05		8.80E+04		1.30E+04		2.76E+03	

C. Isolation of ZIKV from serum and semen specimens

Background

A subset of 40 specimens was selected to attempt isolation of ZIKV from serum and semen to determine the Real Time RT-PCR range in which they are to be considered likely infectious. In addition, we also attempted to isolate ZIKV from 10 serum samples that were positive for ZIKV by Real Time RT-PCR from the CDC Dengue Branch Surveillance System.

Virus isolation methods

For ZIKV isolation from specimens, we tested 100 µl of serum and 500 µl of semen specimens. Specimens were diluted to 1mL with serum-free M199 media and inoculated onto a monolayer of Vero cells in a 25-cm² flask. Infections were performed for 2 hours at 37°C with gentle rocking every 15 minutes. After the infection period, the inoculum was removed and fresh M199 media with 2% heat-inactivated serum was added. Inoculated flasks were incubated at 37°C and observed daily for cytopathic effect. Supernatants were passaged up to two times every seven days. The CDC Trioplex Real Time RT-PCR Assay was performed on supernatants from every passage. If after three weeks in culture, no cytopathic effect or positive RT-PCR was obtained, the specimen was considered negative for ZIKV isolation. Successfully isolated viruses displayed both a complete cytopathic effect of the cell monolayer and decreased CT values with each passage.

Results

ZIKV isolation was attempted in 20 study semen specimens with CT values ranging from 19 to 37, and 20 serum specimens from the ZiPer with CT values ranging from 21-37. Virus isolation was successful in 3 semen specimens with CT values ranging from 19 to 27, and 1 serum specimen with a CT value of 20. We also attempted to isolate ZIKAV from 10 non-ZiPer study serum specimens with CT values ranging

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from 20-33 during the validation of the isolation assay. ZIKV was successfully isolated from 4 non-study
serum specimens with CT values ranging from 20 to 27.

Conclusion

We were able to isolate ZIKV from a small subset of serum and semen specimens. Our results suggest
that specimens from patients that have $CT \leq 27$ in semen or $CT \leq 27$ in serum in the CDC Real Time RT-
PCR should be considered infectious.

D. Modelling approach

Weibull model details

We fit separate parametric Weibull accelerated failure time (AFT) survival models to the outcomes of the time to loss of RNA detection in serum, urine, and semen, and the time to detection of IgM. The outcome of time to loss of RNA detection in each fluid was defined as the DPO until first negative RT-PCR result. For those intermittently shedding, we used the first negative result after the final recorded RT-PCR-positive test result. Time to IgM detection was defined as the DPO until first IgM-positive result. These models followed the standard Weibull AFT model form of:

$$S(t) = e^{-\lambda t^p}$$

For which p is the Weibull 'shape parameter'. The parameter λ is expressed in terms of regression coefficients in the form of the Weibull 'scale parameter', $\frac{1}{\lambda^{1/p}} = e^{\alpha + \sum \beta}$. Models included only an intercept predictor and were thus of the form $\frac{1}{\lambda^{1/p}} = e^{\alpha}$.¹

Survival time estimates at given quantiles of cumulative survival (ie: 50th and 95th percentiles) were found by solving for t :

$$t = (-\ln(S(t)))^{1/p} \times e^{\alpha}$$

Using SAS PROC LIFEREG, standard maximum-likelihood estimation (MLE) approaches were to estimate the regression coefficient α and the shape parameter p and their asymptotic-normal standard errors, which were used per the above equation to estimate survival times at quantiles of cumulative survival and their asymptotic-normal 95% confidence intervals.²

Inclusion of censored observations

The maximum-likelihood estimation approach utilized by SAS PROC LIFEREG enables the partitioning of the likelihood to for two important forms of censoring present in our data: 1) interval censoring due to loss of RNA detection (or IgM seroconversion) between study visits (ie: exact event times not known) and 2) right-censoring of participants with ongoing RNA detection or lack of IgM detection, due to the interim nature of the analysis (ie: not all participants have yet completed follow-up), and loss-to-follow-up. Both sources for right-censoring were assumed uninformative with respect to the detection outcomes.^{2,3}

Assessment of Weibull distribution assumption

Model fit for each specimen type was assessed using the standard graphical approach based on plots of $\ln(-\ln(\hat{S}(t)))$ vs. $\ln(t)$. Inspection for linearity of each plotted curve was used to assess appropriateness of the Weibull distribution assumption.¹

Supplemental models

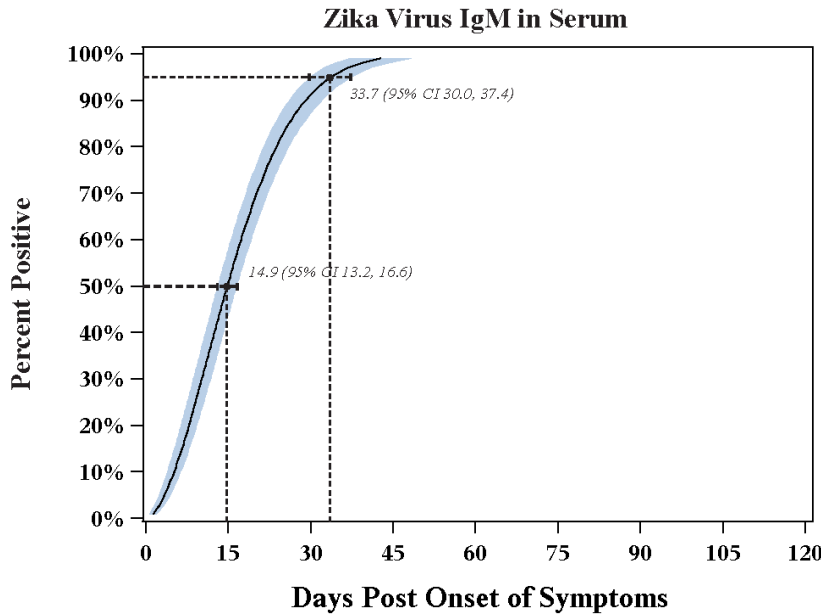
In addition to primary analysis based on the entire prospective cohort, this supplement provides results from alternative models that applied the same Weibull modeling approach to two subsets of participants: 1) those who ever had detection of RNA in the given body fluid analyzed and 2) the set of index participants, thus excluding the 19 symptomatic household contacts included in the prospective cohort.

References

1. Kleinbaum DG, Klein M. Survival Analysis: A Self-learning Text. 3 ed. New York, NY: Springer; 2012.
2. SAS/STAT® 13.1 User's Guide: The LIFEREG Procedure. Cary, NC: SAS Institute Inc.; 2013.
3. Kalbfleisch JD, Prentice RL. The Statistical Analysis of Failure Time Data. New York, NY: John Wiley & Sons; 1980.

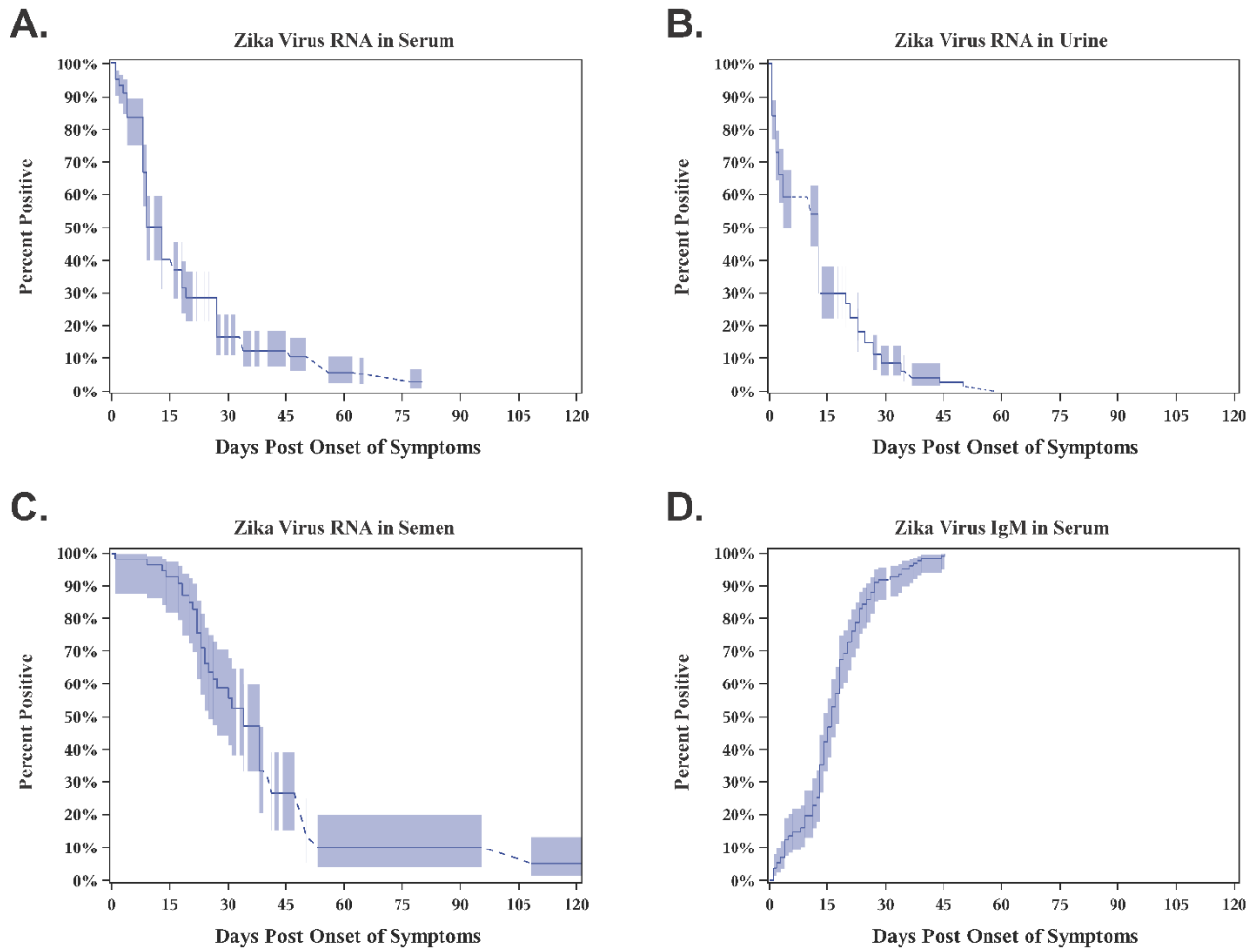
D. Figures

Figure S1. Weibull model-estimated time to detection of anti-Zika virus IgM antibody among all symptomatic participants, Zika Virus Persistence Study, Puerto Rico, 2016



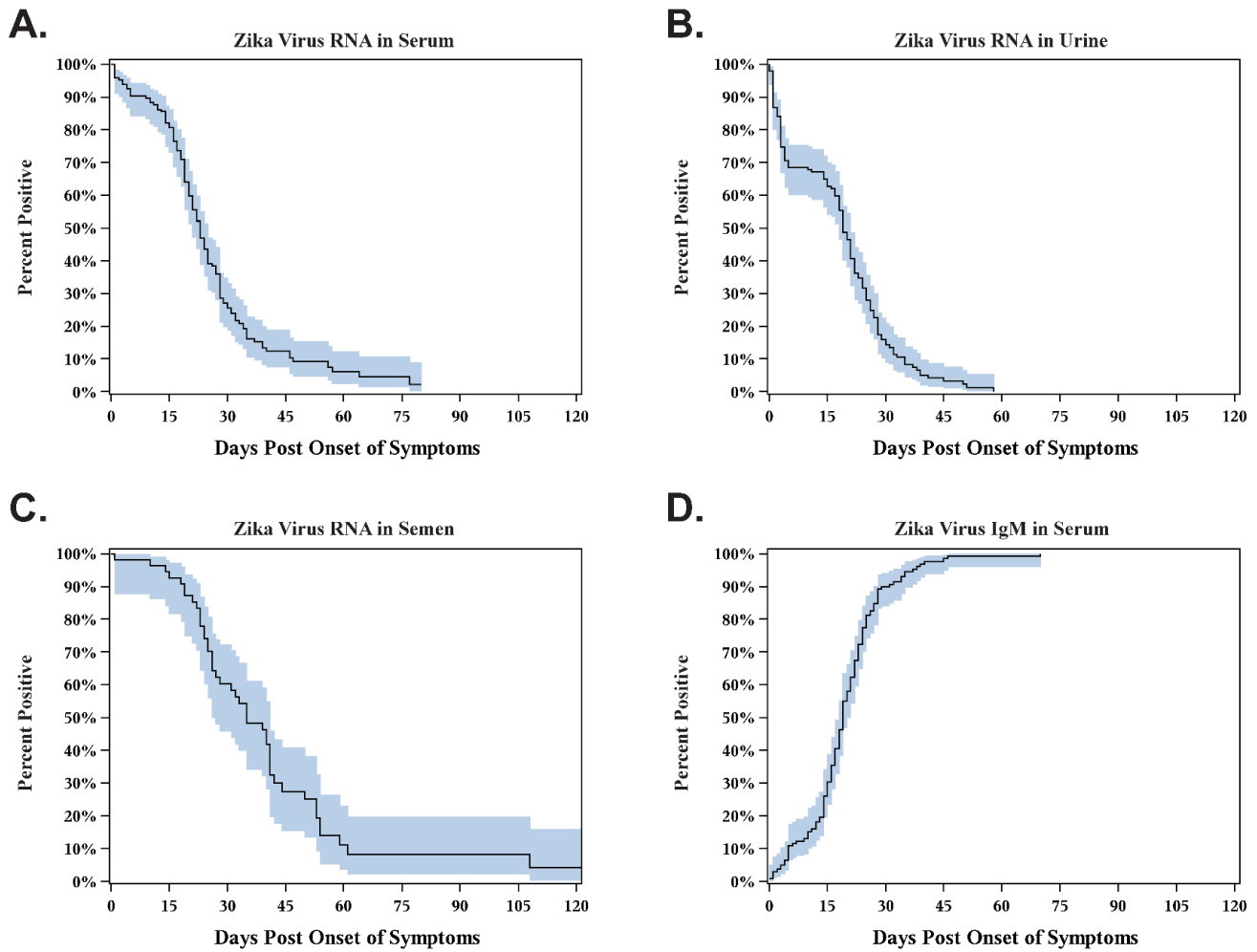
Note: Model of time to first anti-Zika IgM detection in serum was estimated using Weibull regression. Median and 95th percentile and 95% Confidence Intervals (blue shading) shown in figure.

Figure S2. Time to clearance of Zika virus RNA and detection of anti-Zika virus IgM antibody among all symptomatic participants, Turnbull method, Zika Virus Persistence Study, Puerto Rico, 2016



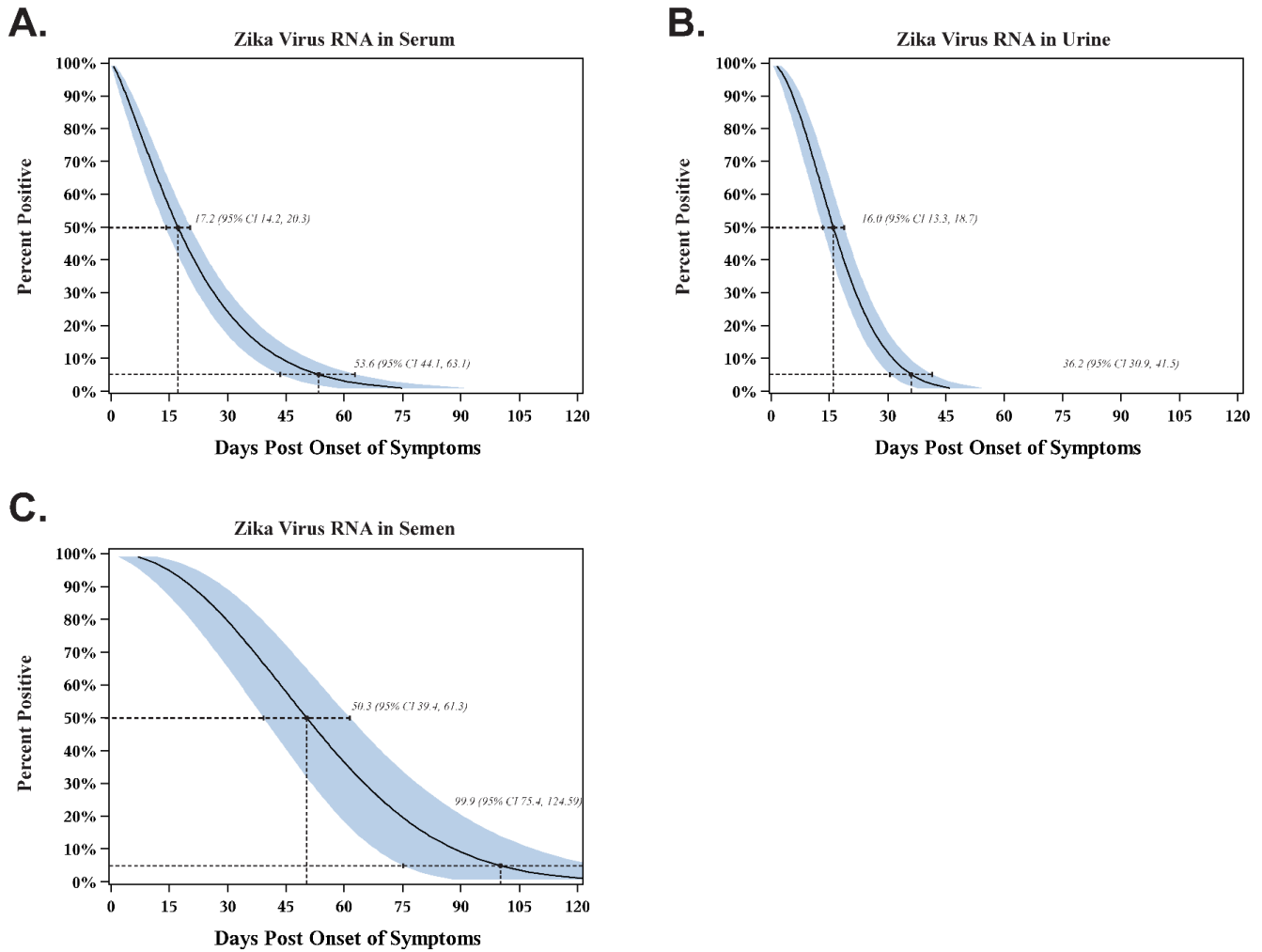
Note: For this method, clearance and detection times reflect days post onset. Turnbull models accounted for interval censoring. Median times to loss of ZIKV RNA detection were 13 days (95% Confidence Interval [CI]:9-13) for serum (A), 13 days (95% CI:4-13) for urine (B), 34 days (95% CI:25-38) for semen (C) and 16 days (95% CI:14-18) for IgM (D). Blue shading denotes 95% CI.

Figure S3. Time to clearance of Zika virus RNA and detection of anti-Zika virus IgM antibody among all symptomatic participants, Kaplan-Meier method, Zika Virus Persistence Study, Puerto Rico, 2016



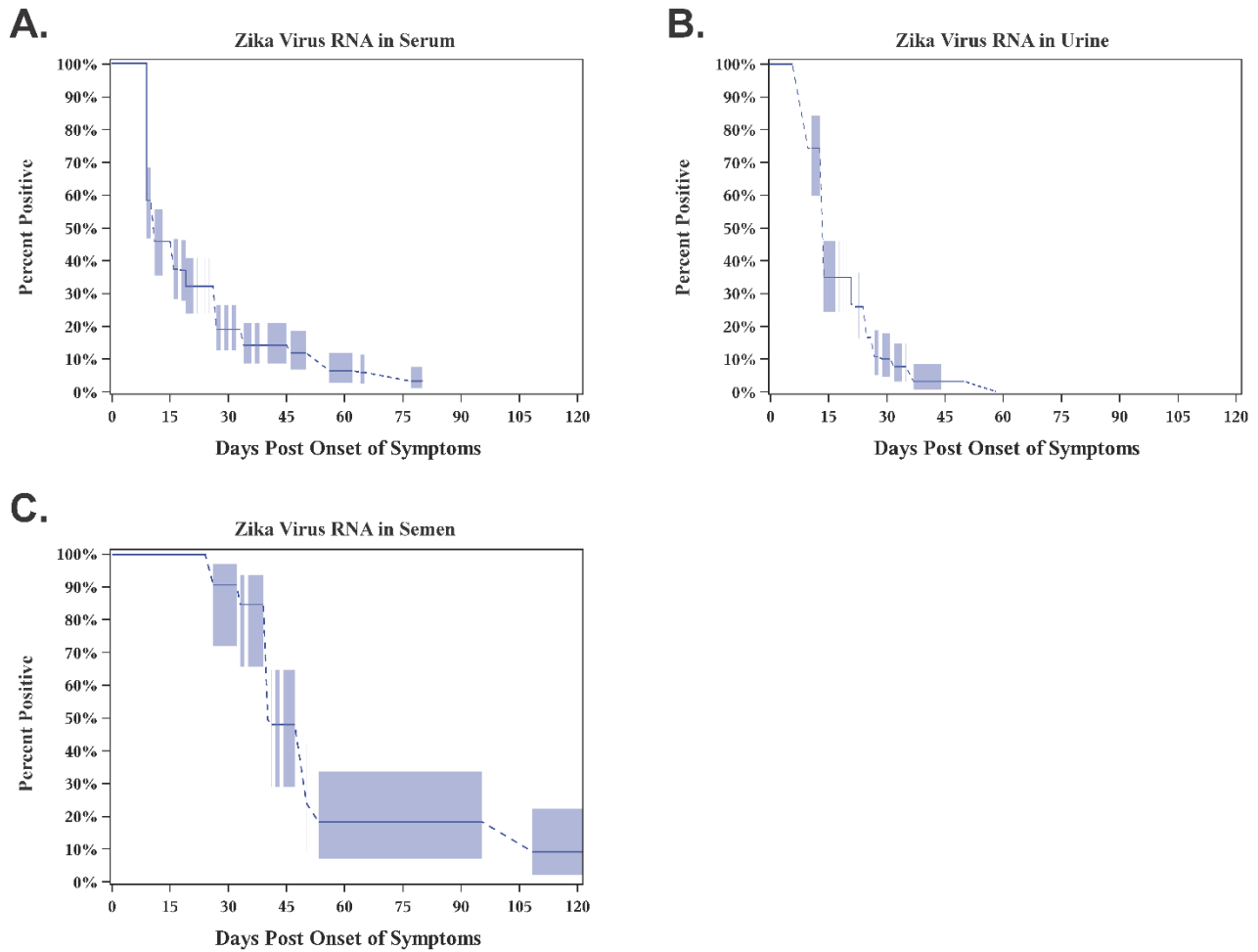
Note: For this method, clearance and detection times reflect days post onset at study visits and do not account for interval censoring. Weibull and Turnbull estimates account for censoring. Results are presented for serum (A), urine (B), semen (C) and IgM in serum (D). Blue shading denotes 95% Confidence Intervals.

Figure S4. Weibull model-estimated time to clearance of Zika virus RNA among participants with detectable Zika virus RNA, Zika Virus Persistence Study, Puerto Rico, 2016



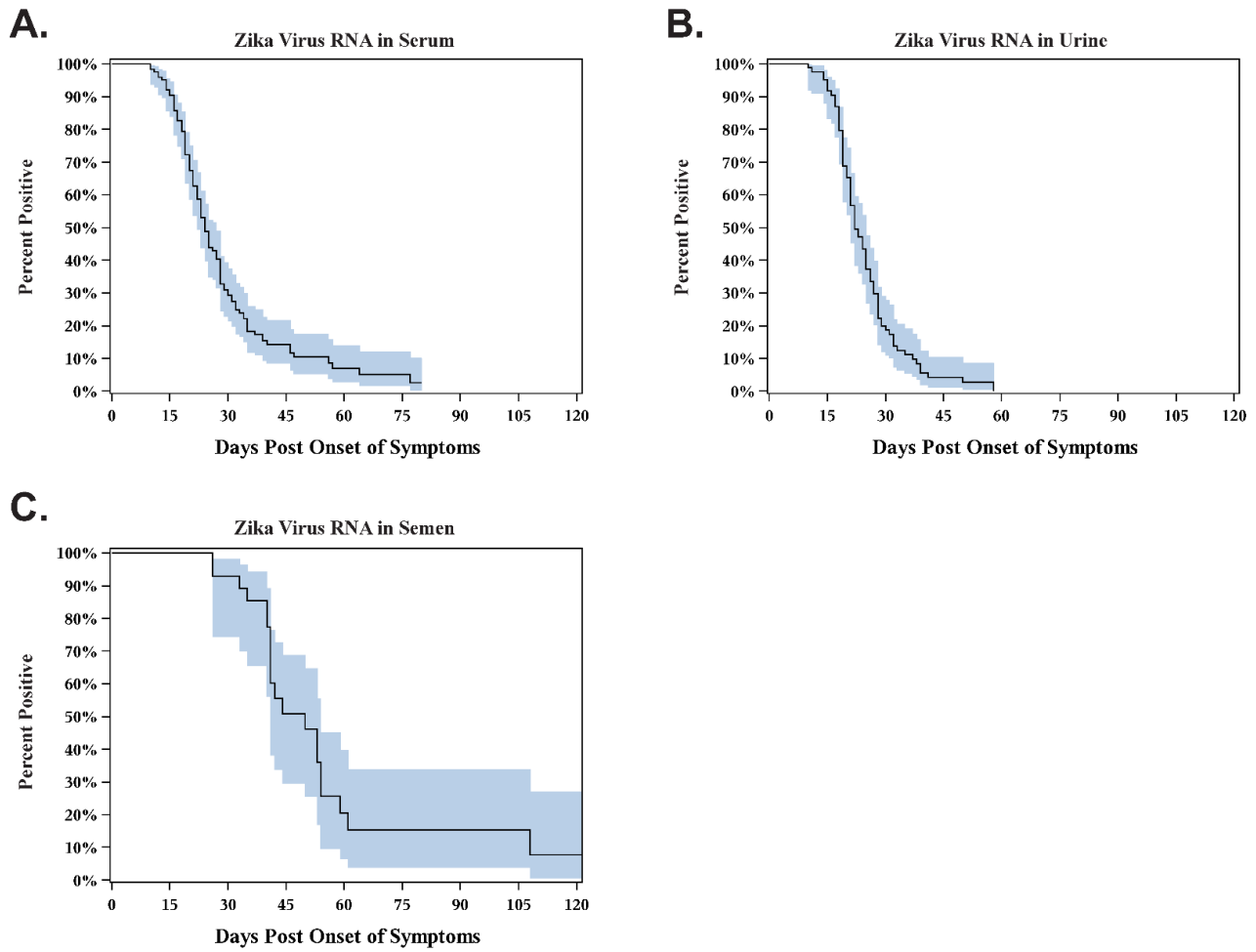
Note: Weibull models accounted for interval censoring. Results are presented for serum (A), urine (B), semen (C). Blue shading denotes 95% Confidence Intervals.

Figure S5. Time to clearance of Zika virus RNA in serum, urine and semen among participants with detectable ZIKV RNA, Turnbull method, Zika Virus Persistence Study, Puerto Rico, 2016



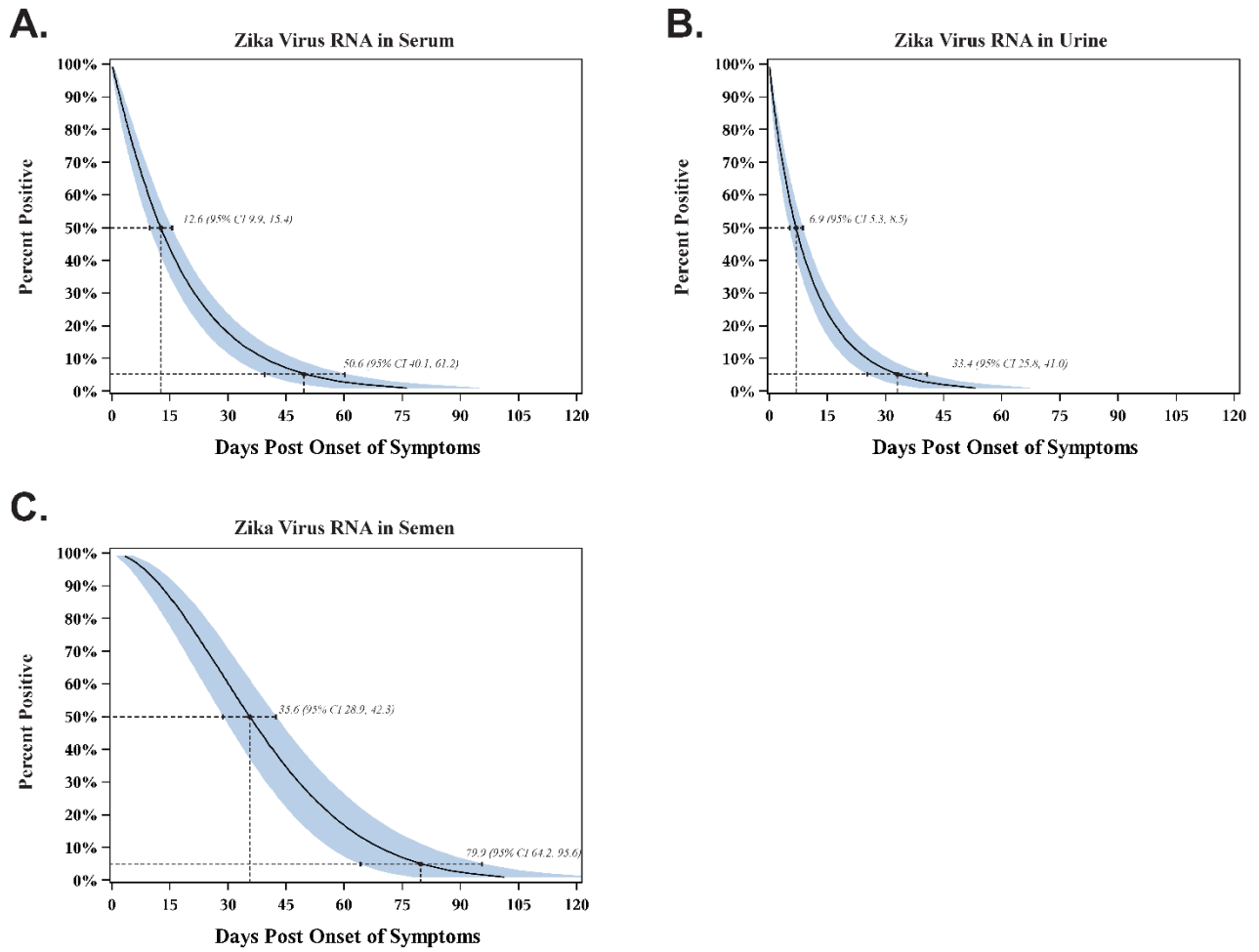
Note: For this method, clearance and detection times reflect days post onset. Turnbull models accounted for interval censoring. Median times to loss of ZIKV RNA detection were 11 days (95% Confidence Interval [CI]:9-16) for serum (A), 13 days (95% CI: undefined) for urine (B), 40 days (95% CI:40-50) for semen (C). Blue shading denotes 95% CI.

Figure S6. Time to clearance of Zika virus RNA in serum, urine and semen among participants with detectable ZIKV RNA, Kaplan-Meier method, Zika Virus Persistence Study, Puerto Rico, 2016



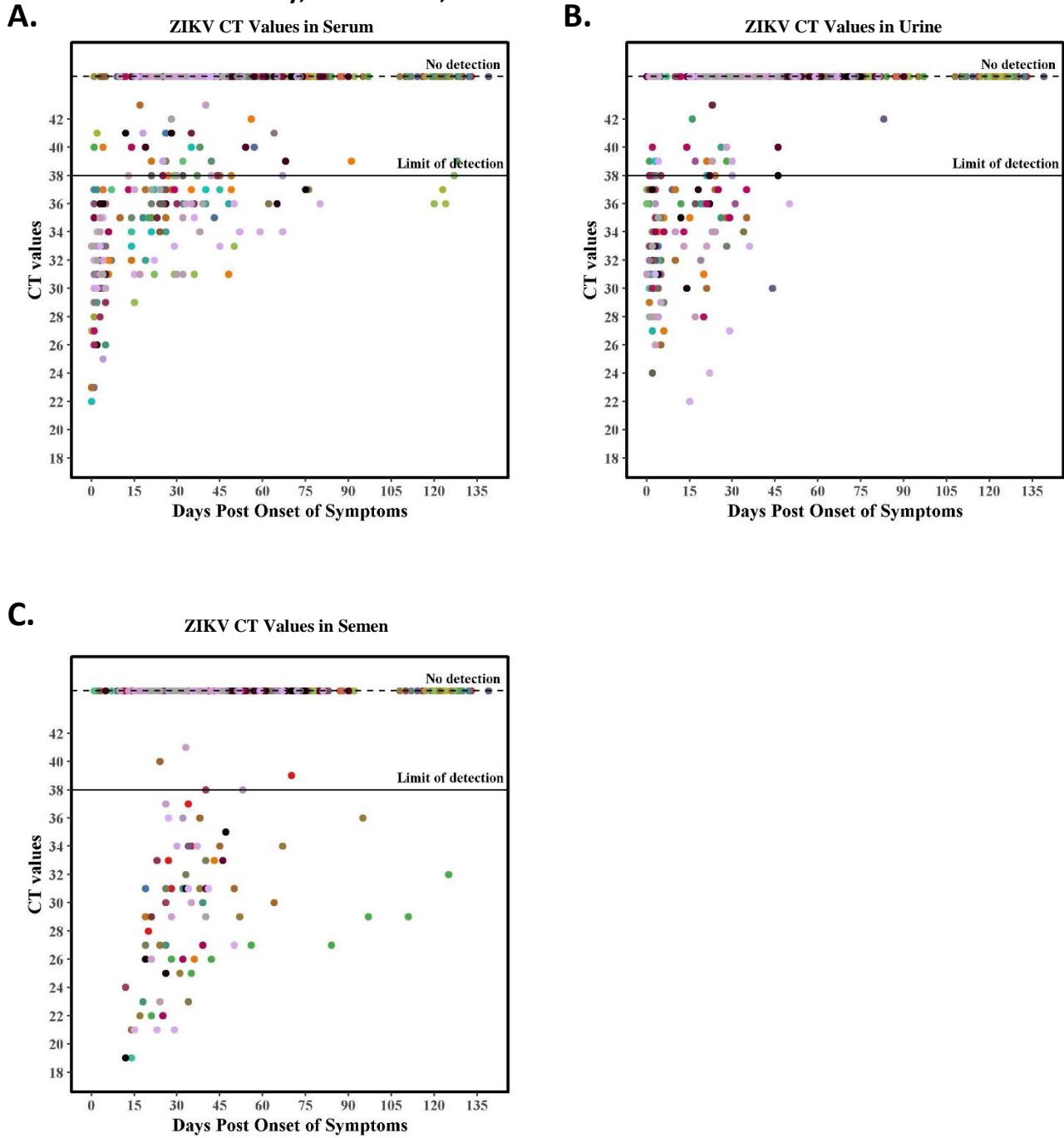
Note: For this method, clearance and detection times reflect days post onset at study visits and do not account for interval censoring. Weibull and Turnbull estimates account for censoring. Results are presented for serum (A), urine (B), and semen (C). Blue shading denotes 95% Confidence Intervals.

Figure S7. Weibull model-estimated time to clearance of Zika virus RNA among index case-patients only, Zika Virus Persistence Study, Puerto Rico, 2016



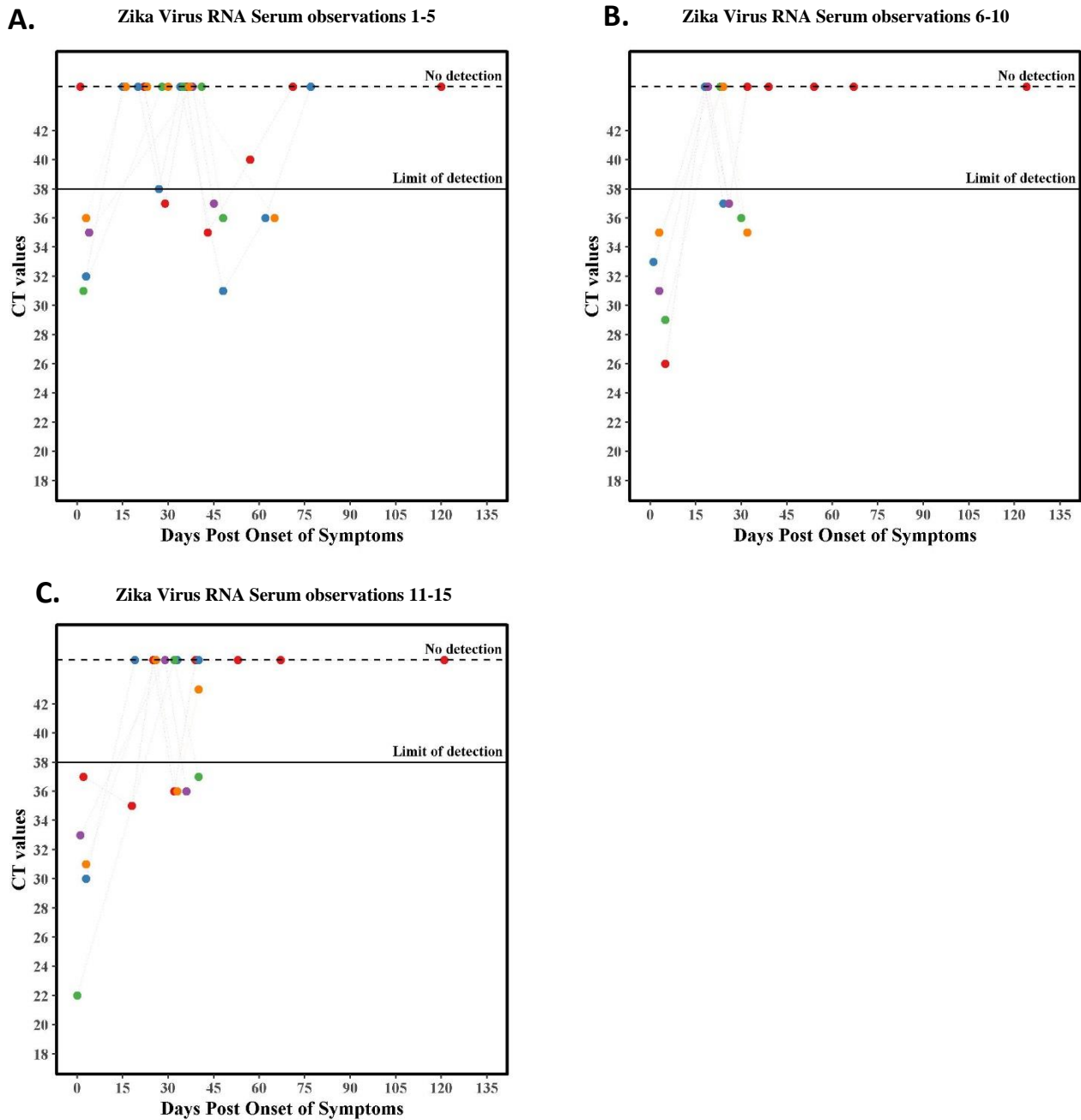
Note: Weibull models accounted for interval censoring. Results are presented for serum (A), urine (B), semen (C). Blue shading denotes 95% Confidence Intervals.

Figure S8. Cycles of amplification by days after the onset of symptoms in serum, urine and semen, Zika Virus Persistence Study, Puerto Rico, 2016



Note: Specimens were considered positive if target amplification was detected within 38 amplification cycles (CT). Specimens were considered negative if target amplification was not detected or detected after 38 amplification cycles. Results are presented for serum (A), urine (B), and semen (C). For saliva, the median CT value among positive specimens was 33 (minimum 20, maximum 37). For vaginal swabs, the CT value for the only positive result was 35.

Figure S9. Cycles of amplification by days after the onset of symptoms among participants with intermittent ZIKV RNA detection in serum, Zika Virus Persistence Study, Puerto Rico, 2016



Note: Specimens were considered positive if target amplification was detected within 38 amplification cycles (CT). For clarity, each panel (A, B and C) presents results for five participants (observations) each. Intermittent ZIKV RNA detection in serum could be due to detection of leftover particles of virus, virus re-activation, or due to re-infection with ZIKV. Zika virus infection is expected to confer long term protection in immunocompetent people. Re-infection with ZIKV seems an unlikely explanation since animal models suggest that primary Zika virus infection elicits protective immunity. Re-infection has been rarely reported for other flaviviruses such as dengue virus. For the estimation of the main study outcome (time to loss of RNA detection), we used the first negative result after the final recorded RT-PCR-positive test result for individuals with intermittent RNA detection. This approach resulted in

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longer estimates of loss to RNA detection, compared to what may be concluded from cross-sectional assessments that do not have the advantage of repeated specimens from the same individual, or even from longitudinal studies that stop follow-up at the first negative result. Current diagnostic guidance recommends to conduct RT-PCR on serum specimens collected within two weeks after symptom onset. Our results support these recommendations as half of infected participants had RNA present at 14 days. Although some individuals with intermittent detection of RNA may be missed by PCR testing, all such individuals in this study had detectable IgM antibody, which according to the existing recommendations would have still provided diagnostic evidence of Zika virus infection.