

Disclaimer

This document is a draft and the information contained herein is subject to change as this document is currently undergoing review by the World Health Organization Ethical Review Committee.

The final version of this standardized protocol: *Prospective longitudinal cohort study of Zika-infected patients to measure the persistence of Zika virus in body fluids* will be published as soon as the ethical review has been completed.

Standardized Protocol:

Prospective longitudinal cohort study of Zika-infected patients to measure the persistence of Zika virus in body fluids

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This protocol outline is based on Zika virus persistence protocol generated by the Ministry of Health Brazil, Fiocruz Research Institutes, Walter Reed Army Institute of Research, Pan American Health Organization/World Health Organization.

Collaborators from Institut Pasteur, the World Health Organization (WHO), and members of the Consortium for the Standardization of Influenza Seroepidemiology (CONSIDE) adapted this protocol as a generic tool for research of Zika virus (ZIKV) infection. A large number of individuals were involved in the content and revision of this protocol and are listed at the end of the protocol.

More information on CONSIDE can be found on their [website](#).

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PROTOCOL SUMMARY

The World Health Organization (WHO) and Pan American Health Organization (PAHO), Institut Pasteur, the networks of Fiocruz, the Consortium for the Standardization of Influenza Seroepidemiology (CONSIDE), the International Severe Acute Respiratory and Emerging Infection Consortium (ISARIC) and many other international research groups have generated standardized clinical and epidemiological research protocols and questionnaires to address key public health questions for Zika virus (ZIKV).

The geographic scope of the current ZIKV outbreak is vast, extending throughout the Americas and the Caribbean and into parts of Africa. The use of standardized research protocols can ensure that results from these studies can be compared across regions and countries and will potentially improve the quality of observational studies by identifying and minimizing biases.

The standardized protocol described below has been designed to maximize the likelihood that epidemiological, clinical and exposure data and biological samples are systematically collected and shared rapidly in a format that can be easily reproducible, aggregated, tabulated and analyzed across many different settings globally. We encourage all study centers to contribute to this effort regardless of resource availability or patient volume, but the ownership of the primary data remains firmly with the individual countries and study sites.

The protocol described below is a prospective longitudinal cohort study, designed to collect data to estimate the risk of conditions related to a pathogen in a population. This standardized study protocol outlines methods to follow a cohort of ZIKV-infected patients to measure the persistence of ZIKV in body fluids. The data collected from this standardized protocol will be used to refine and update recommendations for surveillance to help understand spread, severity and spectrum of the disease and to adapt public health measures, especially for pregnant women and couples planning a pregnancy.

Other protocols currently under development include:

- Case-control study to assess potential risk factors related to microcephaly including Zika virus infection
- Case-control study to assess potential risk factors related to Guillain-Barré Syndrome including Zika virus infection
- Prospective longitudinal cohort study of women and newborns exposed to Zika virus during the course of pregnancy
- Prospective longitudinal cohort study of newborns and infants born to mothers exposed to Zika virus during pregnancy
- Cross-sectional seroprevalence study of Zika virus infection in the general population
- Clinical characterization protocol for Zika virus infection in the context of co-circulating arboviruses

Study groups may decide to implement several protocols during a ZIKV epidemic. In this case, participants may be enrolled in several studies (e.g. cohort of pregnant women and ZIKV persistence study). However, each study group needs to consider carefully the burden on each participant.

Comments for the user's consideration are provided in purple text throughout the document, as the user may need to modify methods slightly as a result of the local context in which this study will be carried out.

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LIST OF ABBREVIATIONS

Ab	Antibody
Ag	Antigen
CHIKV	Chikungunya virus
CMV	Cytomegalovirus
CONSIDE	Consortium for the Standardization of Influenza Seroepidemiology
DE	Design effect
HIV	Human Immunodeficiency virus
HSV	Herpes Simplex virus
ICC	Intraclass correlation
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IHR	International Health Regulations
IRB	Institutional Review Board
ISARIC	International Severe Acute Respiratory and Emerging Infection Consortium
LCMV	Lymphocytic choriomeningitis virus
ME	Margin of error
PAHO	Pan American Health Organization
PBMC	Peripheral Blood Mononuclear Cell
PRNT	Plaque-reduction neutralization test
RNA	Ribonucleic acid
RPR	Rapid plasma reagin
RT-PCR	Reverse transcriptase-polymerase chain reaction
SST	Serum separator tube
TORCHS	Toxoplasmosis, other (e.g. varicella), Rubella, Cytomegalovirus, Herpes, HIV, Syphilis
TPHA	Treponema pallidum hemagglutination assay
VZV	Varicella zoster virus
WHO	World Health Organization
WMA	World Medical Association
YFV	Yellow Fever virus
ZIKV	Zika virus

1.0 INTRODUCTION

In the months that have followed the WHO declaration of a Public Health Emergency of International Concern on 1st February 2016, increasing evidence of the association between exposure to ZIKV in pregnant women and microcephaly and other congenital defects in the fetus, has been published (Besnard, Eyrolle-Guignot et al. 2016, Brasil, Pereira et al. 2016, Broutet, Krauer et al. 2016, Cauchemez, Besnard et al. 2016, Driggers, Ho et al. 2016, Kleber de Oliveira, Cortez-Escalante et al. 2016, Mlakar, Korva et al. 2016, Musso and Gubler 2016, Schuler-Faccini, Ribeiro et al. 2016). ZIKV RNA has now been found in the brains of infants born with microcephaly as well as in the amniotic fluid of mothers exposed to ZIKV during pregnancy (Calvet, Aguiar et al. 2016, Oliveira Melo, Malingier et al. 2016). This implies that, as has been seen with other infections (i.e., TORCHS [toxoplasmosis, other (e.g., varicella, etc.), rubella, cytomegalovirus, herpes, HIV, syphilis]) which can be transmitted mother-to-child and which cause congenital defects such as microcephaly, there is a range of possible effects, in addition to microcephaly, due to ZIKV exposure during pregnancy (Jones, Lopez et al. 2003, Naing, Scott et al. 2016, Yazigi, De Pecoulas et al. 2016).

Limits to knowledge on ZIKV biology in general and its pathogenesis in humans remain. ZIKV has been detected in blood, urine, semen, cerebral spinal fluid, saliva, amniotic fluid, and breast milk (Mlakar, Korva et al. 2016, Barzon, Pacenti et al. 2016, Dupont-Rouzeyrol, Biron et al. 2016, Bonaldo, Ribeiro 2016). In most ZIKV infected individuals, the virus is detected in the blood from several days to one week after the onset of symptoms. ZIKV has also been found to persist longer in urine and semen, and sexual transmission of ZIKV has been recently documented (D’Ortenzio, Matheron et al. 2016, Turmel, Abgueguen et al. 2016, Fréour, Mirallié et al. 2016, Musso, Roche et al. 2015, Mansuy, Duterte et al. 2016, Gourinat, O’Connor et al. 2015). Without a more granular understanding of the kinetics of ZIKV infection across biologic compartments, it will be difficult to devise rational measures to prevent the transmission of the virus.

The following prospective cohort study protocol outlines methods to follow a cohort of Zika-infected patients including symptomatic participants with positive reverse transcriptase-polymerase chain reaction (RT-PCR) test in blood and/or urine and their symptomatic or asymptomatic household/sexual contacts with positive RT-PCR in blood and/or urine in order to measure the persistence of Zika virus in body fluids. This study will address the following public health questions:

1. In which body fluids does ZIKV persist?
2. How long can infectious ZIKV be shed from the body fluids of convalescents after the virus is no longer detectable in the blood?
3. What is the risk of sexual transmission of ZIKV?

Comment: Before submission to a local/national Institutional Review Board (IRB), the introduction will need to be updated with the most recent research findings and further description of the epidemiology of the outbreak in the country conducting this study.

1.1 OBJECTIVES

The data collected from this study will be used to refine and update recommendations for surveillance, to help understand spread, severity and spectrum of the disease and to adapt public health measures, especially for pregnant women and couples planning a pregnancy.

The **primary objectives** of this study are to:

- Assess the presence and duration of infectious Zika virus and related markers (Zika virus specific RNA, antigen, antibodies, T cell response and innate immunity) in blood, urine, saliva, oral fluid, semen, sweat, tears, vaginal secretions, menstrual blood, rectal swab, and breast milk of infected individuals who present to clinics during acute illness and convalescence
- Compare the presence and duration of infectious Zika virus of infected individuals to their infected or uninfected household/sexual contacts.

Comment: Prospective longitudinal cohort studies, such as the one described here, provide the opportunity to assess several **secondary objectives**, including, but not limited to:

- Determine the relationship between host and environmental factors (socio-demographics, chronic diseases such as diabetes, hypertension, obesity, cell-mediated immune response and antibody response) as well as co-infections (Dengue, Chikungunya, HIV and syphilis) on the duration of the persistence of the virus in body fluids.
- Assess the proportion of asymptomatic infections among household contact to infected case.
- Assess the possibility of reinfection or reactivation of the virus cases over the study period.
- Describe concordance between RT-PCR test results, neutralizing antibodies, virus isolation, antigen results, and specific ZIKV antibody responses in these fluids.

Comment: Additional secondary objectives may be included in the protocol, as informed by the outbreak characteristics and by the local context.

2.0 STUDY PROCEDURES

Overview: This is a prospective observational cohort study of men and women, aged 18 years and above, who have ZIKV positive RT-PCR blood or urine samples and their symptomatic or asymptomatic household/sexual contacts with positive RT-PCR in blood and/or urine samples.

2.1 STUDY SETTINGS

2.1.1 STUDY AREA

The selection of recruitment sites should consider:

- Evidence of ZIKV circulation and/or recent outbreak
- Strong community health network
- Laboratory facilities able to perform viral culture, ZIKV antigen assays, RT-PCR, IgM/IgG, neutralizing antibodies test (specific for ZIKV, dengue and chikungunya), HIV testing (Ab or Ab/Ag) syphilis (RPR, TPHA) and genetic sequencing of ZIKV

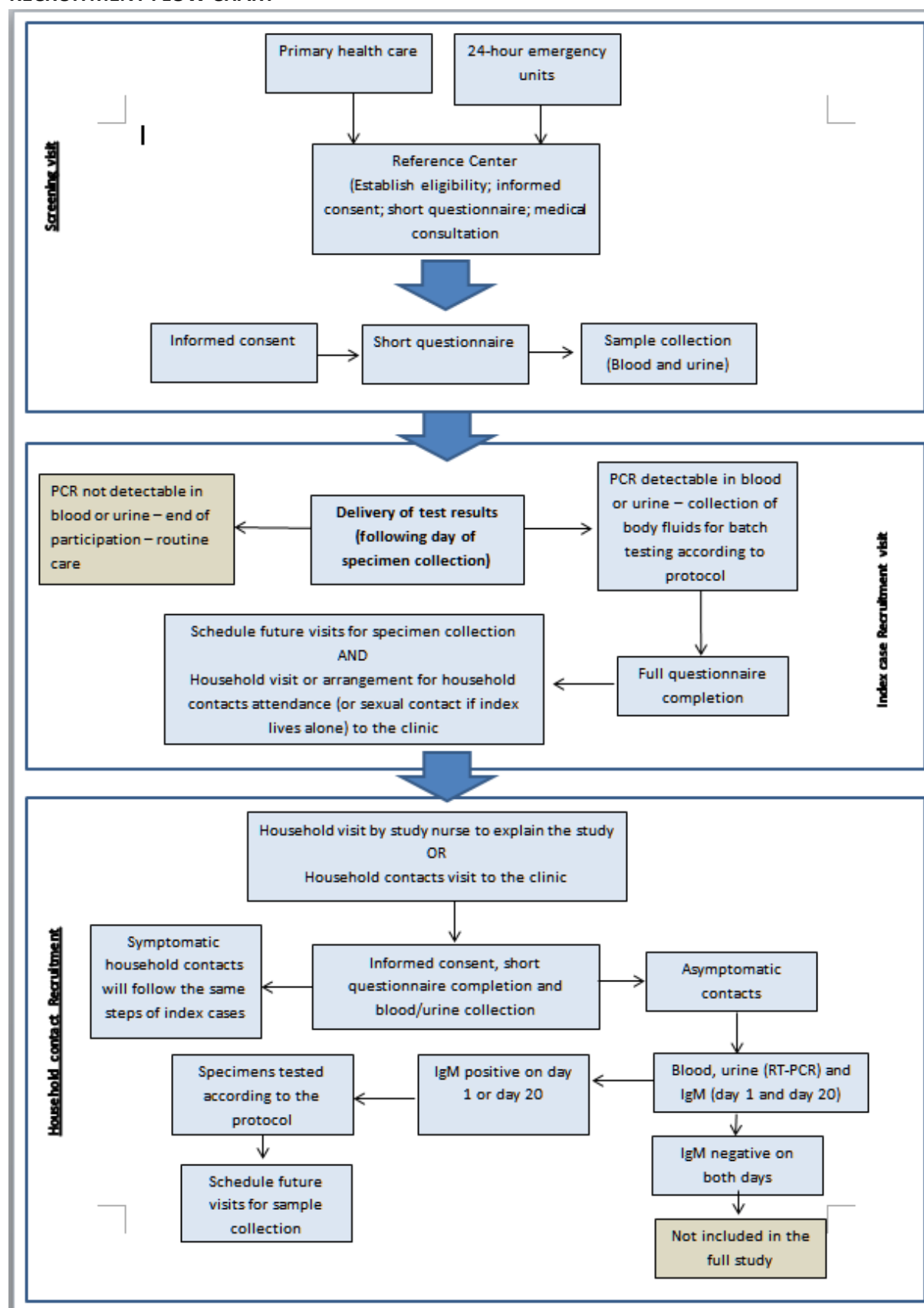
2.2 SELECTION AND RECRUITMENT OF STUDY PARTICIPANTS

2.2.1 STUDY POPULATION

Selection of index cases: This study will include symptomatic men and women (including pregnant women) aged 18 years and above with ZIKV positive RT-PCR blood or urine samples. Potential index case participants will be identified as those who seek clinical care at health care facilities identified by the study group.

Selection of household/sexual contacts: A household will be defined as all persons who occupy a particular housing unit as their usual residence or who live there at the time of the disease of the case. Sexual contacts up to 14 days before the onset of symptoms in the index case will be considered if the index cases lives alone. Household/sexual contacts will be tested at the health care facility where the index case was diagnosed. All household/sexual contacts aged 18 years and above with ZIKV positive RT-PCR blood or urine samples will be considered as potential household/sexual contact participants.

RECRUITMENT FLOW CHART



2.2.2 PARTICIPANT FOLLOW-UP SCHEDULE:

Recruitment and baseline visit: All men and women aged 18 years or above presenting with ZIKV symptoms seeking clinical care at health care facilities are eligible to be enrolled in the study. The study will be explained to potential participants and informed consent will be signed. A short demographic and virus exposure questionnaire will be followed by body fluid specimen collection. For individuals with positive RT-PCR for ZIKV in blood or urine samples, a list of household contacts will be recorded.

If the index case lives alone, a list of sexual contacts will be recorded. Household/sexual contacts will be tested for ZIKV and those with a positive RT-PCR test for ZIKV in blood and/or urine samples will be invited to join the study. The study will be explained to household/sexual contacts and informed consent will be signed. A short demographic and virus exposure questionnaire will be followed by body fluid specimen collection.

Follow-up: Index case participants who consent and have yielded a positive result for ZIKV infection will be followed for 12 months in order to evaluate for persistence of virus, reactivation and reinfection at 2, 4, 10, 20, 30, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330 and 360 days following the baseline visit and specimen collection. At each follow up visit, the relevant questionnaire and body fluid specimen collection will be completed.

Household/sexual contact participants who consent will be followed for 12 months in order to evaluate for transmission of the virus at 3, 5, 11, 21, 30, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330 and 360 days following the baseline visit and specimen collection. At each follow up visit, the relevant questionnaire and body fluid specimen collection will be completed.

2.2.3 ELIGIBILITY CRITERIA

Inclusion criteria:

Index case: Any symptomatic men and women (including pregnant women) aged 18 years and above with ZIKV positive RT-PCR blood or urine samples.

Household contact: Any symptomatic or asymptomatic men and women (including pregnant women) aged 18 years and above who occupy the same housing unit as an index case or who live there at the time of the disease of the case and who have a positive RT-PCR test for ZIKV in blood and/or urine samples.

Sexual contact: Any symptomatic or asymptomatic men and women (including pregnant women) aged 18 years and above who have had a sexual relationship with the index case in the 2 weeks

before the onset of disease in the index case and who have a positive RT-PCR test for ZIKV in blood and/or urine samples.

For the index case, household contact and sexual contact: Permanence for 12 months at a place that allows sample collection for this period.

Exclusion criteria: Any man or woman under the age of 18 years old, or who is unable or unwilling to give informed consent, or with any contraindication to venipuncture, or who will immediately relocate outside the study area.

Comment: The inclusion or exclusion of pregnant women should be carefully considered.

2.2.4 INFORMED CONSENT

Written informed consent will be collected from all study participants, or from a proxy if necessary.

During the first interview with a potential index case, the purpose of the study will be explained and written informed consent will be obtained from the participant upon enrollment into the study by a trained member of the investigation team. Each study participant will be informed that his or her participation is voluntary and that he or she will be free, without justification, to withdraw from the study at any time without consequences. Data contributed to the study up until the point of withdrawal will remain with the study group, unless stated by the withdrawing participant.

Informed consent will seek approval to collect socio-demographic information, blood, urine, saliva, gingival crevicular, semen, sweat, tears, vaginal secretions, menstrual blood, rectal swab, and breast milk samples, as appropriate, for the intended purpose of the study, the possibility that samples may be shipped outside of the home country for additional testing and/or analysis and that samples may be used for future research purposes.

Comment: The study group will need to define the parameters of data sharing with partners outside the country and of future research for which the samples may be used.

Informed consent will also indicate that any suspected or confirmed ZIKV infection may be notified to national authorities under the International Health Regulations (IHR) requirements.

If the study participant agrees, the consent form must be completed legibly, with both surname and first name, dated and signed by the participant and the member of the investigation team, before any procedure can be performed as part of the current study. The member of the investigation team is responsible for obtaining the written consent of the participant.

Once the informed consent form has been signed, one copy will be made and given to the study participant. The original version of the consent form for each participant will be retained by the investigation team and kept in a secure place for a period of time determined by national/local IRB requirements.

Information for participant and informed consent form template can be found in Appendix A.

2.2.5 INCENTIVES TO PARTICIPATE AND COMPENSATION

The primary benefit of this study is the frequency of testing of people with potential exposure to ZIKV, which will allow for timely detection of any abnormality or risk and for appropriate decision-making. Indirectly, the data collected in this study will help improve and guide efforts to prevent the spread of the virus. All study participants will also be provided with additional information by trained social and healthcare workers on means of protection against ZIKV vectors, on other potential modes of ZIKV transmission and on the risk of microcephaly.

The possibility to offer financial compensation (e.g., for expenses to attend medical visits) will depend on the context of the study and local policies should be determined on a study-by-study basis. This will need to be detailed in the information provided to the participant and in the informed consent.

Comment: The clinical management of patients is not a part of this research protocol. It will be at the discretion of the medical consultant and carried out according to standard of care at the site at which recruitment occurred.

2.2.6 POLICY ON INCIDENTAL FINDINGS

Unexpected incidental findings not related to ZIKV may be identified during the course of the study. In this context, the study participant and/or parent/guardian will be informed and, with their consent, a referral will be made to an appropriate clinic or health facility for further investigation or longer-term follow-up. Patient confidentiality will be maintained throughout the study.

2.3 ETHICAL CONSIDERATIONS

Ethical approval will be sought in accordance with local, regional and national authorities. The sponsor and the investigators will be committed to conducting this research in accordance with the [World Medical Association \(WMA\) Declaration of Helsinki](#) (Ethical Principles for Medical Research Involving Human Subjects) adopted by the 64th WMA General Assembly, Fortaleza, October 2013.

Comment: The seven standardized protocols are being submitted for approval to the [Ethics Review Committee](#) of the World Health Organization.

Comment: The study group will need to indicate which IRB has approved the adapted protocol, including the date of ethical approval.

2.3.1 BENEFITS/RISKS FOR STUDY PARTICIPANTS

The primary benefit of this study is the frequency of testing of people with exposure to ZIKV, which will allow for timely detection of any abnormality or risk and for appropriate decision-making.

Indirectly, the data collected in this study will help improve and guide efforts to prevent the spread of the virus.

All biological specimens will be collected in accordance with routine medical procedures and will follow normal standards of practice. All risks associated with biological specimen collection will be explained in accordance with normal practice for the health-care facility.

The collection of a small amount of blood, urine, saliva, gingival crevicular fluid, semen, sweat, tears, vaginal secretions, menstrual blood, rectal swab, and breast milk, in order to test for ZIKV exposure during each study visit poses minimal risk to study participants. Specimen collection may cause minor discomfort, pain, or local infection. In particular, vaginal and rectal swabs can, on rare occasions, cause local trauma or infection, the consequences of which are usually minor in nature.

Participants will be informed of their individual results (e.g., if they have evidence of infection with ZIKV or any other relevant infection). Results of any testing are the property of each participant and should be provided to each participant as promptly as possible.

Comment: The implemented protocol and accompanying informed consent must explain the tests that will be performed on any samples collected, how the results of these tests will be used and how they will be delivered to the participants. This will likely depend on local IRB requirements.

Prevention of ZIKV infection and treatment following ZIKV testing will follow national/WHO guidelines, which may be updated:

- In the event of negative ZIKV test, current information on protection from ZIKV infection will be provided.
- In the event of positive ZIKV infection in a woman of child-bearing age, the woman – and her partner, if she wishes – should receive accurate and evidence-based current information on the potential impact of ZIKV infection on her pregnancy [likelihood of any abnormality related to ZIKV infection].

Comment: Additional information on management of positive ZIKV infection during pregnancy provided by the World Health Organization: [Interim guidance - Pregnancy management in the context of Zika virus infection](#) (13 May 2016)

Comment: The study group will need to provide more information to study participants based on the local context and legal setting, as well as details of the counselling services that will be made available to study participants.

2.4 DATA COLLECTION AND MANAGEMENT

2.4.1 DATA COLLECTION

After informed consent is obtained from eligible index cases, a standardized study questionnaire will be administered to all study participants. At enrollment, information to be collected from the **index case and household/sexual contacts** includes:

- Background demographic information, including socioeconomic status, as indicated by wealth index
- Background medical history, background family medical history, and current medical condition, including vaccines received
- Known and potential risk factors (demographic, lifestyle, ecological factors, previous infection etc.) for ZIKV infection, including travel history, vector exposure and vector protection measures
- Basic physical examination by a trained health care worker
- Signs and symptoms of ZIKV infection
- Laboratory evaluations of blood, urine, saliva, gingival crevicular fluid, semen, sweat, tears, vaginal secretions, menstrual blood, rectal swab, and breast milk samples, as appropriate for confirmation of any ZIKV exposure and other relevant infections such as arboviruses (e.g., Dengue) and TORCHS infections [toxoplasmosis, other (e.g., varicella, etc.), rubella, cytomegalovirus, herpes, HIV, syphilis]

At each of the follow-up visits, the following information will be collected from the **index case and household contacts**:

- Clinical data, including any treatments taken or given between follow-up visits,
- Signs and symptoms of ZIKV or other relevant infections (e.g., arboviruses, TORCHS [toxoplasmosis, other (e.g., varicella, etc.), rubella, cytomegalovirus, herpes, HIV, syphilis])
- Laboratory evaluations of blood, urine, saliva, gingival crevicular fluid, semen, sweat, tears, vaginal secretions, menstrual blood, rectal swab, and breast milk samples, as appropriate for confirmation of any ZIKV exposure and other relevant infections such as arboviruses (e.g., Dengue) and TORCHS infections [toxoplasmosis, other (e.g., varicella, etc.), rubella, cytomegalovirus, herpes, HIV, syphilis]
- Recent sexual and reproductive health and sexual activity
- Menstruating women will be asked to provide a specimen as close to the first day of their period as possible, and a second specimen before the end of the period. Menstrual blood sampling may or may not be done at the same time as other body fluid testing. If the woman's period does not coincide with other specimens' collection times, she will be asked to attend a specimen collection on a different day, as appropriate. This will be explained by at the time of the recruitment.

Comment: A standardized questionnaire has been developed specifically for this protocol by the Ministry of Health Brazil, Fiocruz Research Institutes, Walter Reed Army Institute of Research, Pan American Health Organization/World Health Organization.

The questionnaire contains the core data variables that should be collected from the study participants to address the objectives of this study. Further questions may be added at the discretion

of the study group. The questionnaire is designed to be implemented by trained study personnel, without advanced or specialized medical degrees.

2.4.2 DATA MANAGEMENT

All data collected will be stored in databases and all participant identifiable information, such as name and address of each participant, will have an anonymized study ID. The database's location and security requirements will depend on national regulations thereby determined on a study-by-study basis. A password-protected copy of the database will be de-identified (without name, address) and sent for data analysis to the designated data manager(s).

Diagnostic test results will be securely transmitted to the center in charge of data centralization and analysis, which will then be responsible for making the tests results available to the investigation participants. Testing results will be conveyed to participants or to their primary care provider.

Participant identity will be protected and only aggregate summary data released publically. Original data collection forms will be kept in locked storage in accordance with national regulations for an extended period of time after the end of the study. An identification log will be implemented and will be kept in a secure, locked facility within the study country.

Comment: The study group will need to detail procedures for data management, protection and storage in the adaptation of the protocol.

2.5 SPECIMEN COLLECTION AND LABORATORY INVESTIGATIONS

2.5.1 SPECIMEN COLLECTION, STORAGE AND TRANSPORTATION

Blood: to obtain serum, whole blood will be collected through venipuncture in plain vacutainer tubes (or equivalent) allowed to clot and serum separated after centrifugation. Venous blood specimen will be drawn to enable analysis of Zika IgM and IgG antibodies in serum and for the ZIKV Ag assay.

Comment: Ideally, 3x 5mL SST tubes should be collected. However, it is possible to proceed with only 1x 5 mL SST.

To obtain plasma whole blood will be collected through venipuncture in a vacutainer tube containing EDTA, allowed to stand or centrifuge to separate plasma.

For cytokine determination the correct anticoagulant should be heparin.

- 500 µL of fresh specimen will be needed to perform RT-PCR and serology tests.
- 2 aliquots of 1 mL will be frozen at -70 °C for RT-qPCR and serology tests
- 3 aliquots of 2 mL will be frozen at -70 °C for future studies.
- 1 aliquot of 1 mL of serum and 5 ml of peripheral blood mononuclear cell (PBMC) may be shipped.

Urine:

- 10-20 mL of urine will be collected in a screw top urine container
- 3 aliquots of 2 mL of urine will be frozen at -70 °C for RT-qPCR
- 3 aliquots of 2 mL will be frozen for future studies

Oral fluid (saliva, gingival crevicular fluid), semen, sweat, tears, vaginal secretion, menstrual blood, rectal secretion, breastmilk: For these samples, it is important to collect

- at least 500 µL for RT-qPCR and serology (if applicable)
- additional 500 µL kept frozen at -70 °C for future studies.

Participants will be asked for consent to bank their specimens for future research. Specimens of consenting individuals will be kept at -70°C. Specimens that test positive by RT-PCR will also be processed for culture/viral isolation.

Specimen collection: All collection tubes will be labeled with a coded identification number that will also be recorded on the interview questionnaire. Date and time of collection, location, and name of person collecting the specimen will be recorded.

Specimen storage and preservation: Unless specified above, all specimen tubes will be stored temporarily on ice carried by the study teams until they can be transported to the laboratory:

- Refrigerated (2-8° C) if it is to be processed (or sent to a reference laboratory) within 48 hours.
- Frozen (-10 to -20°C) if it is to be processed after the first 48 hours, but within 7 days.
- Frozen (-70 °C) if it is to be processed after a week. The sample can be preserved for extended periods.

If air transportation is needed, ship (insofar as possible) using triple packaging with dry ice within 48 hours. Or, at the very least, maintain the cold chain with cooling gels.

Specimen transportation: Transport of specimens within national borders should comply with applicable national regulations and international transport should comply with applicable [international regulations](#). The original samples will be packed, labeled and marked (if dry ice is used), and documented as Category B.

Comment: The specimen collection and laboratory procedures will need to be clearly defined by the study group.

Comment: At least two aliquots of sample should be made and at least one should be kept for future analysis. As such, specimens may remain in country and only aliquots may be sent to a reference lab, if necessary. This will depend on local IRB requirements.

2.5.2 LABORATORY PROCEDURES

Laboratory testing will be carried out in the country of the research institution collecting biological samples or in collaboration with an external laboratory partner as needed. At least two aliquots of

sample will be made and at least one will be kept for future analysis. The principal tests described for ZIKV infection detection and differential diagnosis are listed in Table 1.

Comment: The list of the laboratory tests and the targeted pathogens provided below may be subject to modifications depending on the local laboratory capacities and circulating pathogens, and thus needs to be considered on a study-by-study basis.

Comment: Yellow fever virus (YFV) may be included in the list of pathogens to investigate in regions in which YFV is currently circulating.

Table 1: *List of the different biological tests to be performed on collected specimens*

Nature of specimen	Lab test	Targeted pathogen	Remarks
Serum	Serology: IgM and IgG	ZIKV, Chikungunya, Dengue, CMV, LCMV, VZV, HSV toxoplasmosis, rubella	If positive result, use same sample for plaque-reduction neutralization test (PRNT)
Urine, oral fluid (saliva, gingival crevicular fluid), semen, sweat, tears, vaginal secretion, menstrual blood, rectal secretion, breastmilk	Real-time RT-PCR	ZIKV, chikungunya, dengue, CMV, LCMV, VZV, HSV toxoplasmosis, rubella	If positive, viral culture may be performed on specimen

Laboratory confirmation of recent ZIKV infection will comprise:

- Presence of ZIKV RNA or antigen in serum or other specimens (e.g. oral fluid, tissues, urine, whole blood); or
- When the patient is found to have nucleic acid specific for ZIKV as well as chikungunya or dengue, confirmatory testing of serum will be required. ZIKV infection will be confirmed when: IgM antibody against ZIKV positive and PRNT90 for ZIKV with titer ≥ 20 and ZIKV PRNT90 titer ratio ≥ 4 compared to other flaviviruses; and exclusion of other flaviviruses. Please see the latest [WHO laboratory guidance for ZIKV](#).
- Virus culture will be performed in order to determine whether a given body fluid sample has live virus

Serological methods: Multiple serological assays may be needed to confirm seropositivity. Indeed, even if antibodies cross-reaction with other genetically related viruses is minimal during primary infection, sera of individuals with a previous history of infection from other flaviviruses (especially dengue, yellow fever and West Nile) may cause cross-reactivity. Although neutralization by plaque reduction (PRNT) offers greater specificity in the detection of neutralizing antibodies (IgG), cross-reactions have also been documented. In fact, some patients with a previous history of infection by other flaviviruses have shown up to a fourfold increase in neutralizing antibody titers when infected

with ZIKV. Thus, primary screening should be performed by enzyme-linked immunosorbent assays, immunoassays or immunofluorescence assays and confirmation will need to include virus neutralization assay.

Molecular methods: The method of choice to detect and quantify the presence of ZIKV particles in body fluids is real-time RT-PCR. Multiple primers specific for ZIKV have been designed by research teams and diagnostic laboratories (see Appendix C for examples of these primers). Commercial kits are also available, but for research use only (Musso & Gubler, 2016). Optimal standardization between laboratories has not yet been achieved. As the choice of primers may depend on the genetic diversity of currently circulating ZIKV strains, adaptation may be required on a study-by-study basis.

Genotyping/selected full-length sequencing: Ideally, additional genomic analysis will be performed on a subset of positive samples by full-length sequencing methods. Whole genome sequencing and cognate viral load measures will be explored. Confirmatory follow-on procedures including real-time quantitative PCR will be performed as necessary, preferably with commercial reagents or alternatively those laboratory developed assays adapted from the literature. Viruses may be obtained by co-culturing with activated PBMCs, if other molecular methods for virus sequence determination fail.

Comment: If this testing cannot be performed in country, samples could be sent overseas for this analysis, depending on local IRB regulations.

Comment: These recommendations are subject to further updates whenever new, reliable diagnostic tests become available for clinical use.

3.0 STUDY ENDPOINTS AND STATISTICAL ANALYSES

3.1 SAMPLE SIZE CONSIDERATIONS

This is a prospective observational cohort study, whose prediction of the exact sample size required cannot be prospectively determined. However, considering the scarcity of existing information on viral shedding, even a few participants will contribute to the body of evidence on the topic and lead to improved public health recommendations.

The following is the rationale for estimating the sample size needed for body fluids other than blood, using a hypothetical estimate of the prevalence of persistence of 50% after 12 months.

Equal sample size in each group was assumed with a two-sided 95% confidence interval width of 10% (or margin of error (ME) of $\pm 5\%$) and 80% power, a sample size of 385 males and 385 females who are either positive for ZIKV in urine or in blood at baseline will be needed.

To account for the design/clustering effect at household level, we applied design effect (DE) of 1.5 assuming a cluster (family) size of 3 ZIKV-infected participants and an assumed an intracluster correlation (ICC) of 0.25, resulting into a sample size of 576. We further assume that 10% of participants, index cases and household contacts, will be lost to follow up, by the end of the first year and hence the final sample size will be 634 males and 634 females. We rounded this number up to 650 each, males and females, 434 households.

The sample size is tentatively estimated, given that there is little information on ZIKV persistence rates in bodily fluids, available in literature. The final sample size of 650 each among males and females is estimated for evaluation of the primary outcome which is the overall ZIKV persistence rate by 12 months, among males and females (taking into consideration a design / clustering effect and loss to follow up). Since the role of different risk factors is not yet well known, the subgroup analyses will be done in an exploratory way and the effect of different factors on the primary outcome will be further explored in multivariable analysis models.

3.2 STUDY OUTCOME MEASURES AND STATISTICAL ANALYSES

The following statistical analyses and primary outcomes correspond to the primary objectives described above. Any secondary outcomes will need to be defined by the research group, as determined by the selection of secondary objectives. Table 2: *Statistical analysis tests to be performed, based on primary objectives*

Objective	Outcome	Statistical analysis
1. Assess the presence and duration of infectious Zika virus and related markers	Detection of ZIKV RNA in body fluid samples at different time points	Summary statistics using mean, median, range of viral load for each body fluid sample. Comparison of mean differences in the viral load between two groups using a t-

		test after log transforming viral load which is positively skewed
2. Compare the presence and duration of infectious Zika virus of infected individuals to their infected or uninfected household contacts	Detection of ZIKV RNA in body fluid samples at different time points in different household contacts	A time to event analysis (Survival analysis) comparing persistence/duration of the virus between the two groups. A log rank test to compare the overall survival functions/curve of the two groups
3. Determine the relationship between host and environmental factors, as well as co-infections on the duration of the persistence of the virus in body fluids.	Demographic, laboratory examination and clinical examination information collected from study participants	Cox regression adjusting for potential confounders and co-infection on the persistence of the virus in body fluids
4. Assess the proportion of asymptomatic infections among household contact to infected case.	ZIKV RNA detection and clinical features in household contacts	A chi-square test to compare symptomatic or asymptomatic infections among those infected and those non-infected
5. Assess the possibility of reinfection or reactivation of the virus cases over the study period	ZIKV RNA detection and clinical features over time	Time to event analysis (Survival analysis)

4.0 REPORTING OF FINDINGS

Reports of the results of this study should follow the 'cohort studies' checklist of the [STROBE statement](#), and include sufficient information to permit pooling of data with similar studies.

Important information to report include (1) the number of index cases recruited, (2) demographic information including age and gender, (3) the number of symptomatic and asymptomatic household contacts recruited and (4) duration and frequency of follow-up tests.

It is also important to fully document the study design, including recruitment methods, the approach to determining ZIKV infection, the laboratory methods used and the outcome measurements.

Ideally, information would be collected in a standardized format and anonymized data would be shared among multiple groups running similar protocols.

5.0 COMPLEMENTARY STUDIES

This protocol address specific questions relative to the potential association between ZIKV infection and congenital abnormalities in the fetus. However, additional aspects of ZIKV infection during pregnancy may be investigated depending on the study context. Complementary studies may therefore be considered in association with this protocol.

Additional standardized protocols for ZIKV are available and include:

- Case-control study to assess potential risk factors related to microcephaly including Zika virus infection
- Case-control study to assess potential risk factors related to Guillain-Barré Syndrome including Zika virus infection
- Prospective longitudinal cohort study of women and newborns exposed to ZIKV during the course of pregnancy
- Prospective longitudinal cohort study of newborns and infants born to mothers exposed to Zika virus during pregnancy
- Cross-sectional seroprevalence study of Zika virus infection in the general population
- Clinical characterization protocol for Zika virus infection in the context of co-circulating arboviruses

6.0 ACKNOWLEDGEMENTS

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A large number of individuals were involved in the creation and revision of this protocol: Adele Benzaken (Ministry of Health, Brazil), Elisete Duarte (Ministry of Health, Brazil), Ana Izabel Menezes (Ministry of Health, Brazil), Silvana Giozza (Ministry of Health, Brazil), Mariana Verotti (Ministry of Health, Brazil), André Abreu (Ministry of Health, Brazil), Thayssa Fonseca (Ministry of Health, Brazil), Ana Bispo (Fiocruz, Rio de Janeiro), Guilherme Calvet (Fiocruz, Rio de Janeiro), Marcus Lacerda (Fiocruz, Manaus), Ana Maraia de Brito (Fiocruz, Recife), Patrícia Brasil (Fiocruz), Patrícia Sequeira (Fiocruz), Camilla Botto de Menezes (Fiocruz), Flor Martinez Espinosa (Fiocruz), Tereza Magalhães (Fiocruz), Kayvon Modjarrad (Walter Reed Army Institute of Research), Lydie Trautmann (Walter Reed Army Institute of Research), Morgane Rolland (Walter Reed Army Institute of Research), Rasmi Thomas (Walter Reed Army Institute of Research), Leandro Sereno (Pan American Health Organization), Ximena Pamela Diaz Bermudez (Pan American Health Organization), Maeve de Mello (Pan American Health Organization), Massimo Ghidinelli (Pan American Health Organization), Ludovic Reveiz (Pan American Health Organization), Nathalie Broutet (World Health Organization), Edna Kara (World Health Organization), Robyn Meurant (World Health Organization), Willy Urassa (World Health Organization), Ndema Habib (World Health Organization), Pierre Formenty (World Health Organization), Sihem Landoulsi (World Health Organization), Anna Thorson (World Health Organization), Joao Paulo Souza (World Health Organization), Maria Van Kerkhove (Institut Pasteur), Rebecca Grant (Institut Pasteur), Sibylle Bernard Stoecklin (Institut Pasteur), and Anna Funk (Institut Pasteur).

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Comment: This list needs to be reviewed, adding individuals and affiliations as appropriate.

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APPENDICES

Appendix A: Description of investigation and informed consent template

Refer to the description of investigation and informed consent developed specifically for this protocol by the Ministry of Health Brazil, Fiocruz Research Institutes, Walter Reed Army Institute of Research, Pan American Health Organization/World Health Organization.

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Appendix B: Standardized questionnaire

Refer to the standardized questionnaire developed specifically for this protocol by the Ministry of Health Brazil, Fiocruz Research Institutes, Walter Reed Army Institute of Research, Pan American Health Organization/World Health Organization.

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Appendix C: List of Published Primers for Detection and Quantification of Zika Virus by Real-time RT-PCR (Cao-Lormeau, Blake et al. 2016)

APPENDIX C: LIST OF PUBLISHED PRIMERS FOR DETECTION AND QUANTIFICATION OF ZIKA VIRUS BY REAL-TIME RT-PCR (CAO-LORMEAU, BLAKE ET AL. 2016)

ZIKV target	Primer/Probe name	Primer sequence	Primer position	Reference
M/E	ZIKV835	TTGGTCATGATACTGCTGATTGC	835-857	(Lanciotti, Kosoy et al. 2008)
	ZIKV911c	CCTTCCACAAAGTCCCTATTGC	911-890	
	ZIKV860F FAM	CGGCATACAGCATCAGGTGCATAGGAG	860-886	
pE	ZIKV1086	CCGCTGCCCAACACAAG	1086-1102	(Lanciotti, Kosoy et al. 2008)
	ZIKV1162c	CCACTAACGTTCTTTGCAGACAT	1162-1139	
	ZIKV1107FAM	AGCCTACCTTGACAAGCAGTCAGACACTCAA	1107-1137	
E	ZIKVENVF	GCTGGDGCRGACACHGGRAC	1538-1558	(Faye, Faye et al. 2008)
	ZIKVENVR	RTCYACYGCCATYTGGRCTG	1902-1883	
NS5	ZIKVF9027a	CCTTGGATTCTTGAACGAGGA	9121-9141	(Balm, Lee et al. 2012)
	ZIKVR9197ca	AGAGCTTCATTCTCCAGATCAA	9312-9290	
NS5	Forward	AARTACACATACCARAACAAAGTGGT	9271-9297	(Faye, Faye et al. 2013)
	Reverse	TCCRCTCCCYCTYTGGTCTTG	9352-9373	
	ProbeFAM	CTYAGACCAGCTGAAR	9304-9320	