



Zika virus and laboratory diagnosis

พิไลพันธ์ พุฒวัฒนะ

ศูนย์วิจัยพัฒนานวัตกรรม

คณะเทคนิคการแพทย์ มหาวิทยาลัยมหิดล

30 มกราคม 2560

Discovery of Zika virus



- Apr 1947: The virus was isolated from sentinel rhesus monkey in Zika forest, Uganda through monitoring of yellow fever.
- Jan 1948: The virus was isolated from *Aedes africanus* mosquito in Uganda
- 1952: Human infections were found in Uganda and Tanzania.

Zika virus outbreaks



- From its discovery until 2007, Zika virus infection were rare in Africa and Southeast Asia.
- 2007- an outbreak occurred in Yap Island, Federation States of Micronesia. **Zika was first considered as an emerging disease.**
- 2013- the outbreak occurred in French Polynesia. Complication with Guillain-Barré syndrome was first noted.



Guillain-Barre syndrome (GBS)

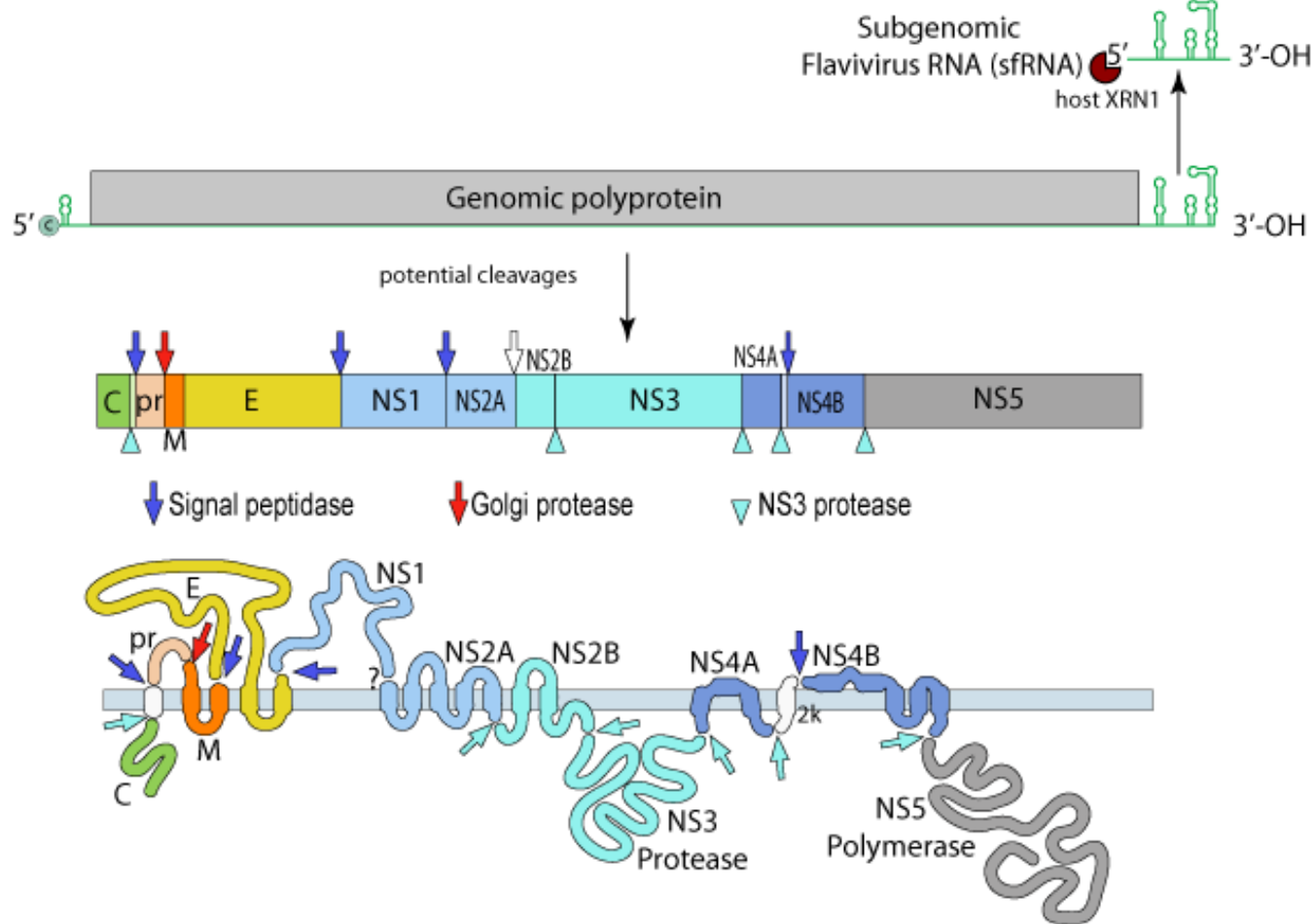
- Acute inflammatory demyelinating polyneuropathy ปลอกหุ้มเส้นประสาทหลายเส้นมีการอักเสบอย่างเฉียบพลัน
- The incidence of GBS during Zika outbreak in French Polynesia was > 20 -folds over the baseline, or approximately 0.24 per 1,000.

May 2015- the outbreak occurred in Brazil.

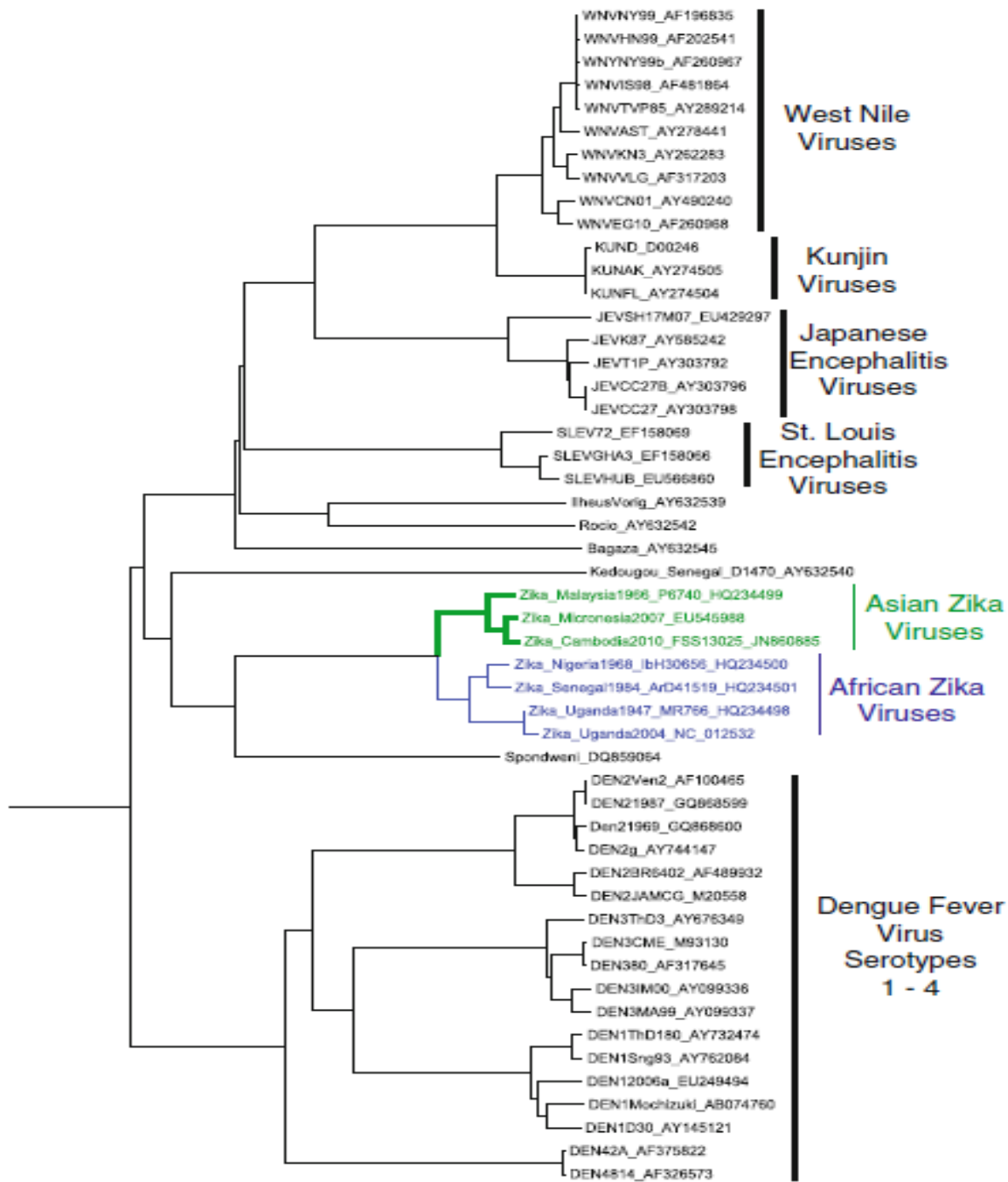
By November 2015, Brazil notified congenital microcephaly that may be associated with Zika virus infection.

On February 1st, 2016, the WHO declared the Zika outbreak as a Public Health Emergency of International Concern (PHEIC).

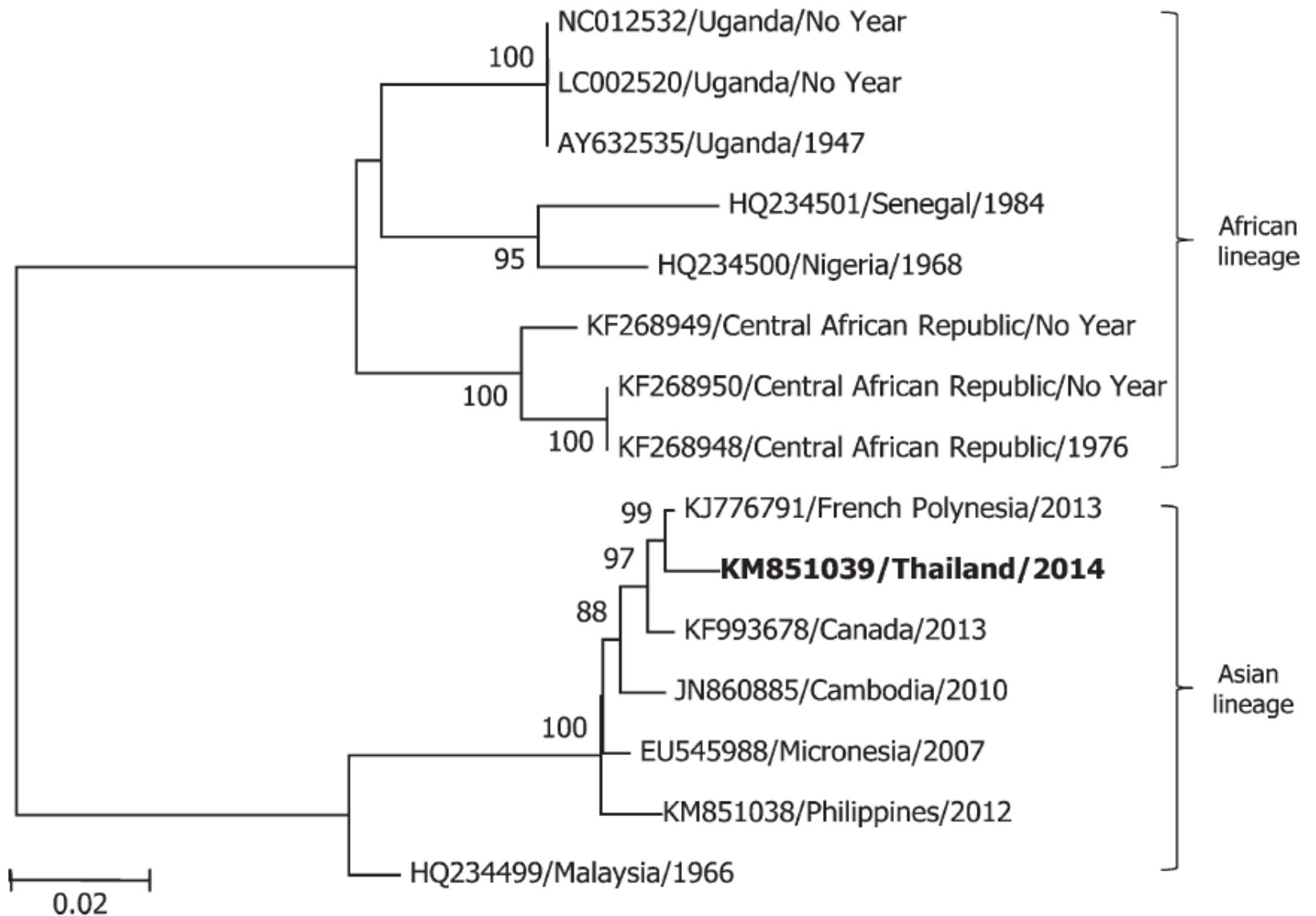




Plus sense, SS RNA genome of 10,794 bp in length and encodes for a polyprotein of 3,419 aa. The polyprotein is processed by host and viral proteases into 3 structural proteins and 7 nonstructural proteins.

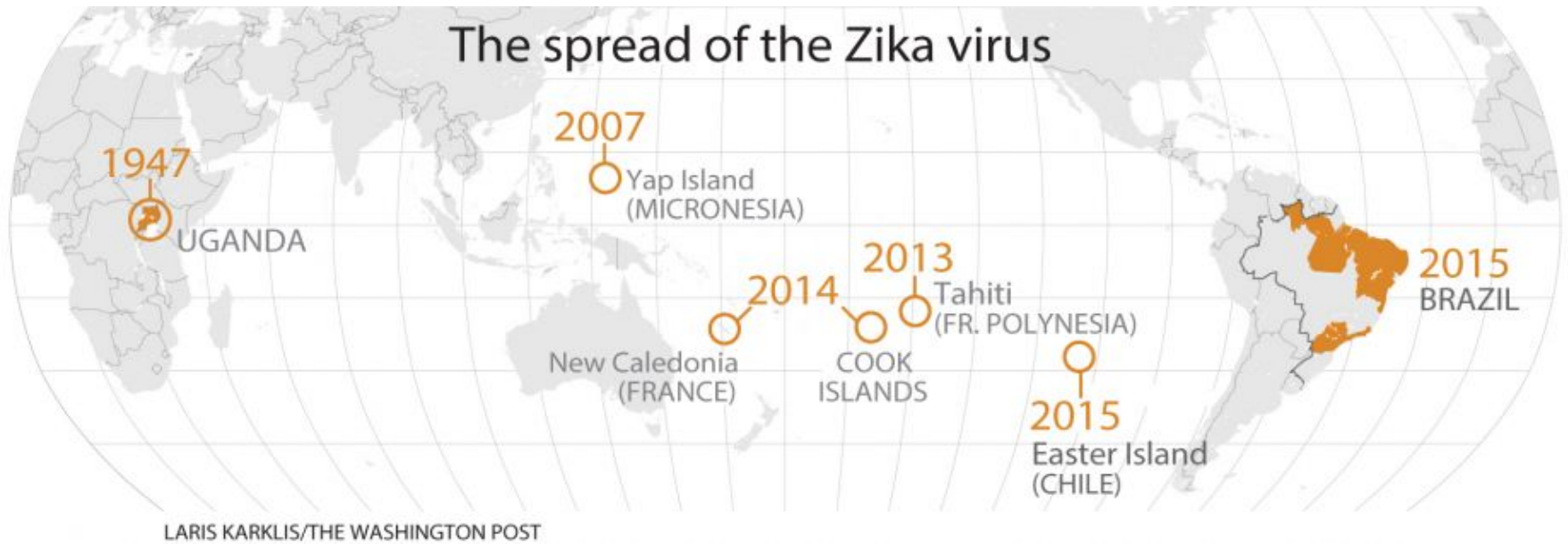


Full genome analysis
Shapschak e al 2015



Phylogenetic trees of fragments of NS5

Buathong R, et al. Am J Trop med Hyg 2015: 93: 380.



<http://www.houstonchronicle.com/news/medical/article/That-little-rash-you-have-Let-s-hope-it-s-not-6522030.php>



Origin of zika lineages

- Both African and Asian lineages emerged in Africa.
- The Asian lineage originated during the virus's migration from Africa to Southeast Asia, where it was first detected in Malaysia (1966?).
- From Malaysia, Zika virus spread to the Pacific Islands, separately to Yap and French Polynesia, and then to New Caledonia, Cook Islands, Easter Island, and the Americas. ???????



- Southeast Asian Zika virus might not be the direct source of South American outbreaks.
- Amino acid residues on envelope, pr and NS1 are unique to South American isolates only.



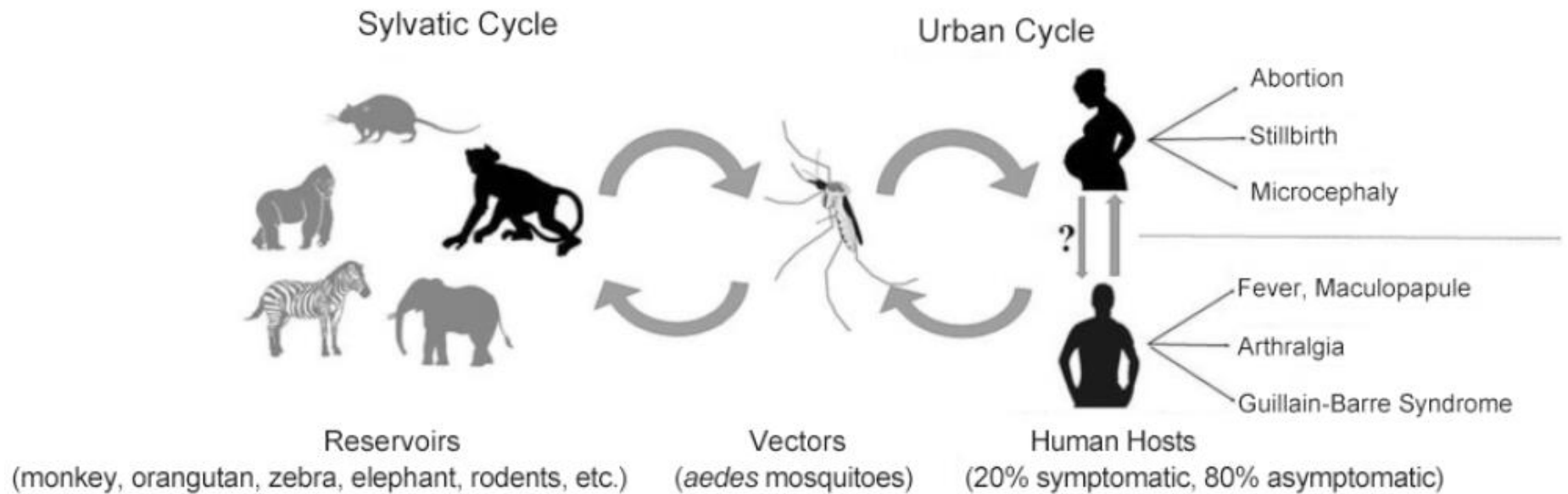
Mosquito vectors

- *Aedes aegypti*, suspected to be major vector in Asia and French polynesia
- *A. albopictus*
- *A. africanus*
- *A. henselli* (outbreak in Yap Island)

Incubation period in mosquitoes is about 10 days.



Transmission cycle of zika virus



Wang, et al Virologic Sinica 2016



Human to human transmission:

- Congenital/intrapartum transmission
- Sexual transmission
- Blood transmission is possible.



Clinical outcome of Zika virus infection

- Incubation period: 3-12 days
- Mostly mild or no symptoms.
- About 25% of infected people develop symptoms, including rash, fever, joint pain, red eyes, and headache.
- Recovery is usually complete and fatalities are rare.



Incubation period

- Extrinsic incubation period in mosquito is about 10 days.
- Intrinsic incubation period in humans is 3-12 days.

SYMPTOMS OF ZIKA VIRUS

HEADACHE

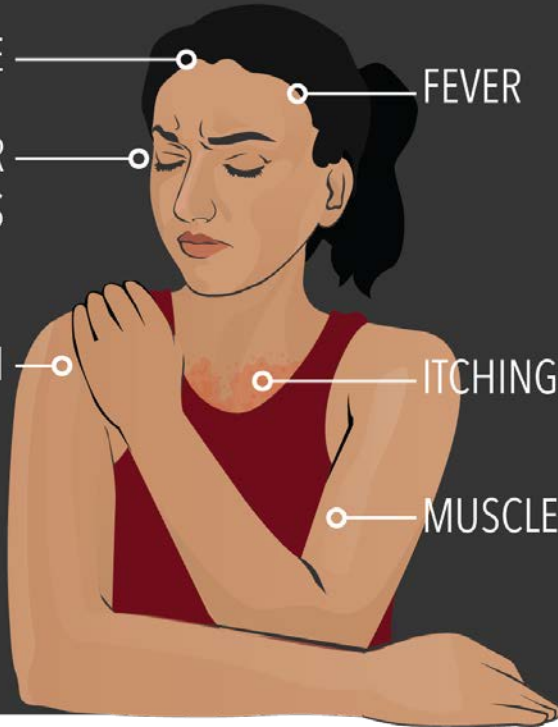
FEVER

PAINFUL OR
RED EYES

JOINT PAIN

ITCHING/RASH

MUSCLE PAIN



[https://en.wikipedia.org/wiki/Zika_virus_outbreak_\(2015-present\)](https://en.wikipedia.org/wiki/Zika_virus_outbreak_(2015-present))

microbewiki.kenyon.edu

Bilateral Conjunctival Hyperemia (Red eyes)

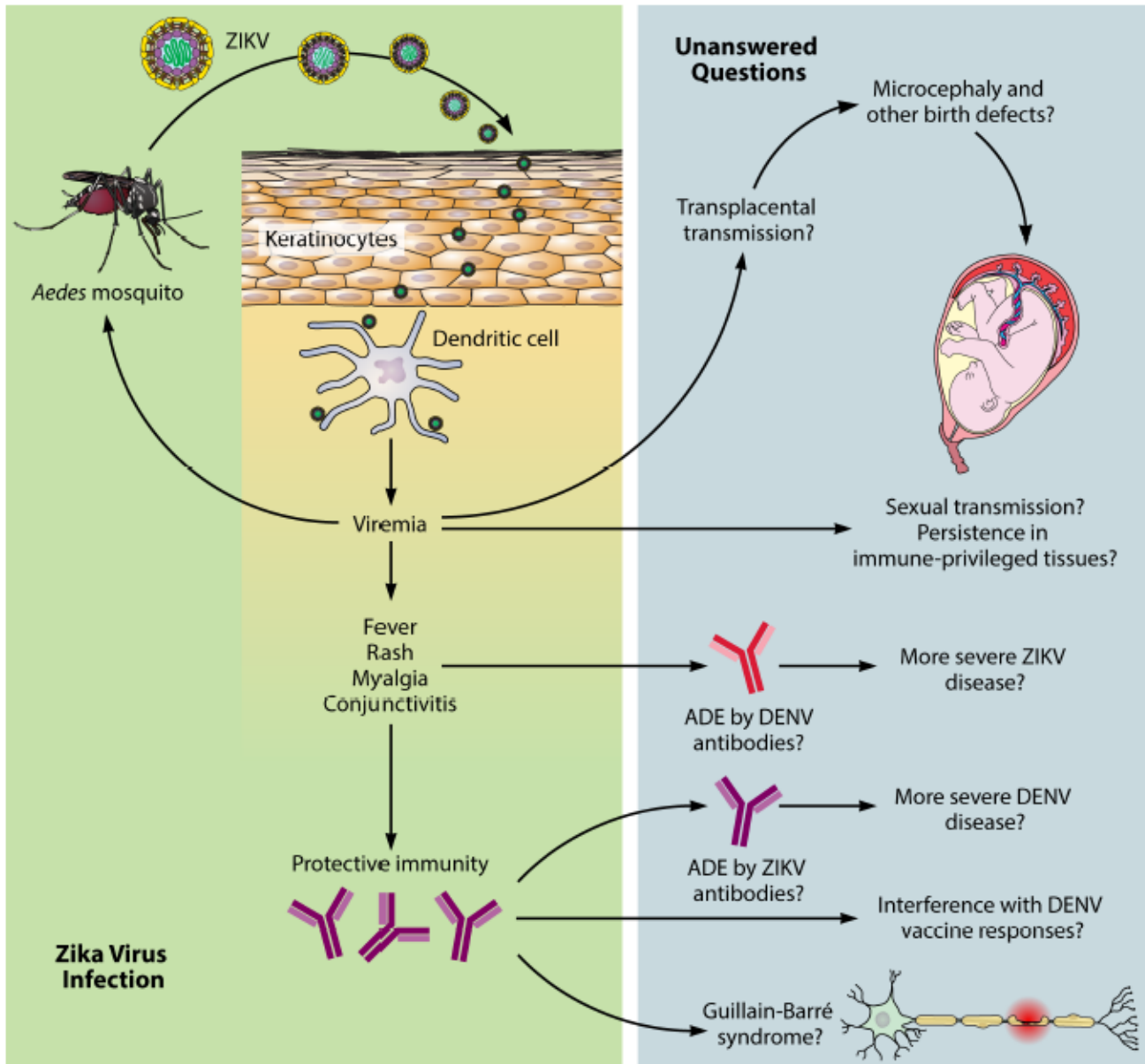


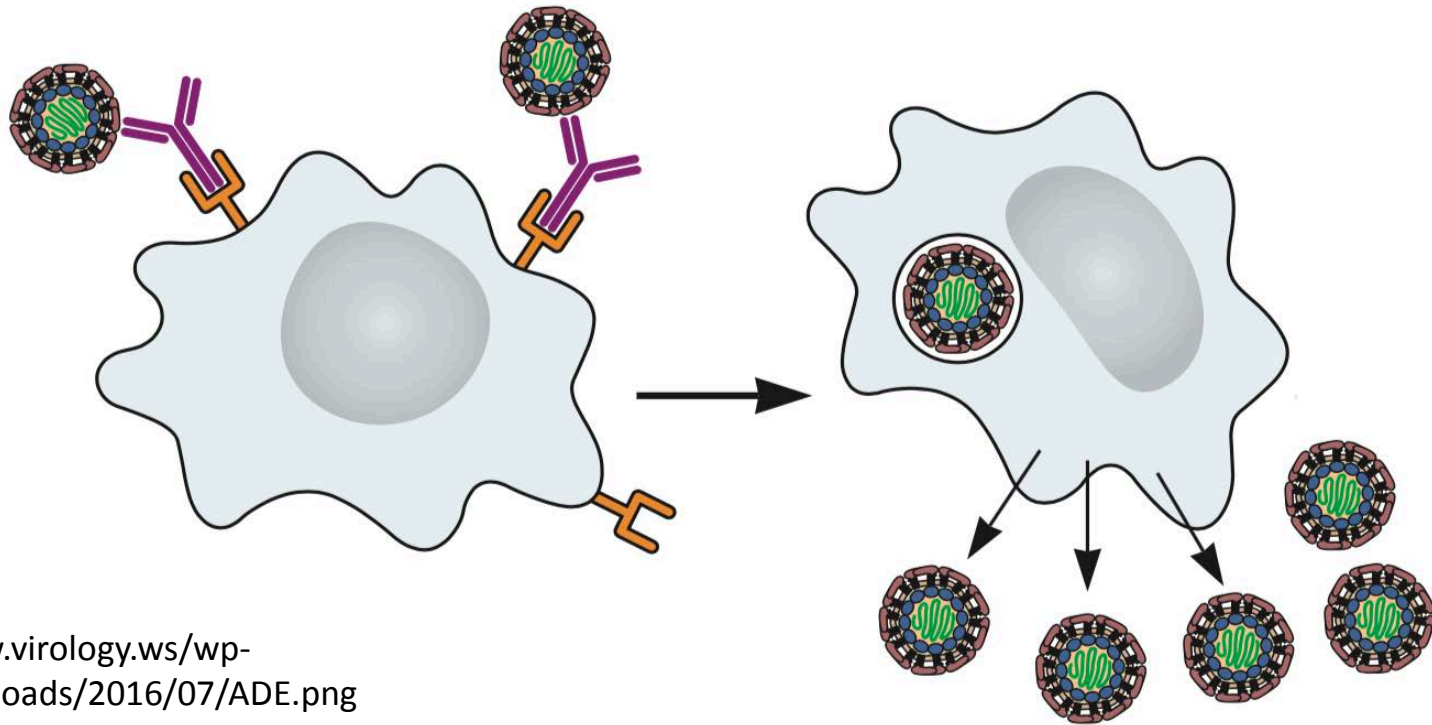
Differential diagnosis



Rickettsial infection and Leptospirosis,
malaria, measles, rubella

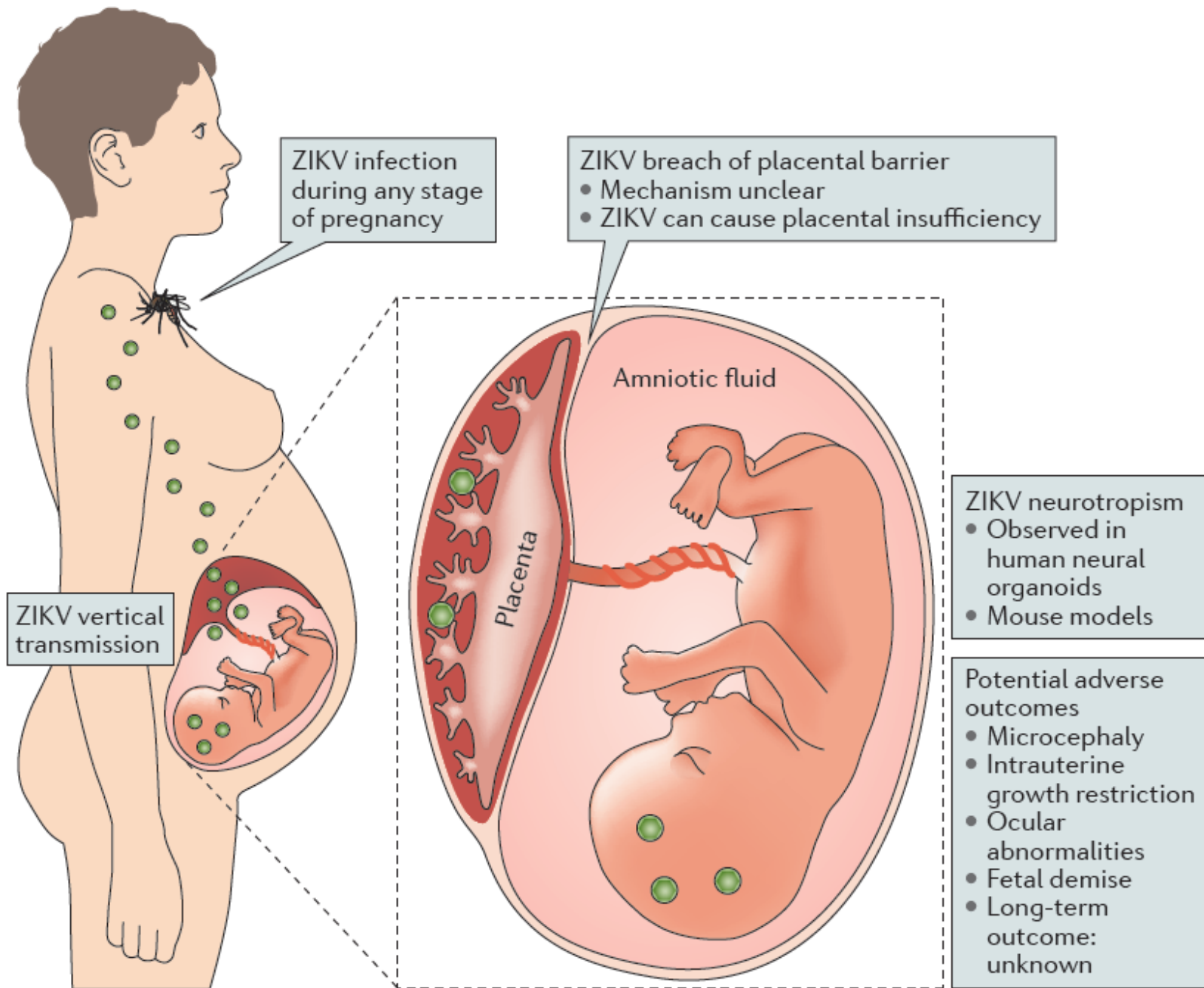
Zika virus pathogenesis





<http://www.virology.ws/wp-content/uploads/2016/07/ADE.png>

Zika differs from DENV by around 41–46% (in the sequence of the envelope protein), the similarities are sufficient to drive antibody dependent enhancement of infection.



MEASURING HEAD CIRCUMFERENCE



Baby with Typical Head Size

Baby with Microcephaly

Baby with Severe Microcephaly

- Use a measuring tape that cannot be stretched
- Securely wrap the tape around the widest possible circumference of the head
 - Broadest part of the forehead above eyebrow
 - Above the ears
 - Most prominent part of the back of the head
- Take the measurement three times and select the largest measurement to the nearest 0.1 cm
- Optimal measurement at 24-36 hours after birth when molding of the head has subsided



Congenital Zika syndrome

In addition to microcephaly, other manifestations include brainstem dysfunction, brain calcification, ocular abnormalities, hearing loss.

Genitourinary, cardiac and digestive systems can be affected

Symptoms are more severe when infection occurs during early gestation.

Zika virus disease – Interim case definition (as of 12 February 2016)

- **Suspected case:** A person presenting with rash and/or fever and at least one of the following signs or symptoms: arthralgia; or arthritis; or conjunctivitis (non-purulent/hyperaemic).
- **Probable case:** A suspected case with presence of IgM antibody against Zika virus (with no evidence of infection with other flaviviruses: and contact with a confirmed case, or a history of residing in or travelling to an area with local transmission of Zika virus within 2 weeks prior to onset of symptom.
- **Confirmed case :** A person with laboratory confirmation of recent Zika virus infection:

Confirmed case : A person with laboratory confirmation of recent Zika virus infection:

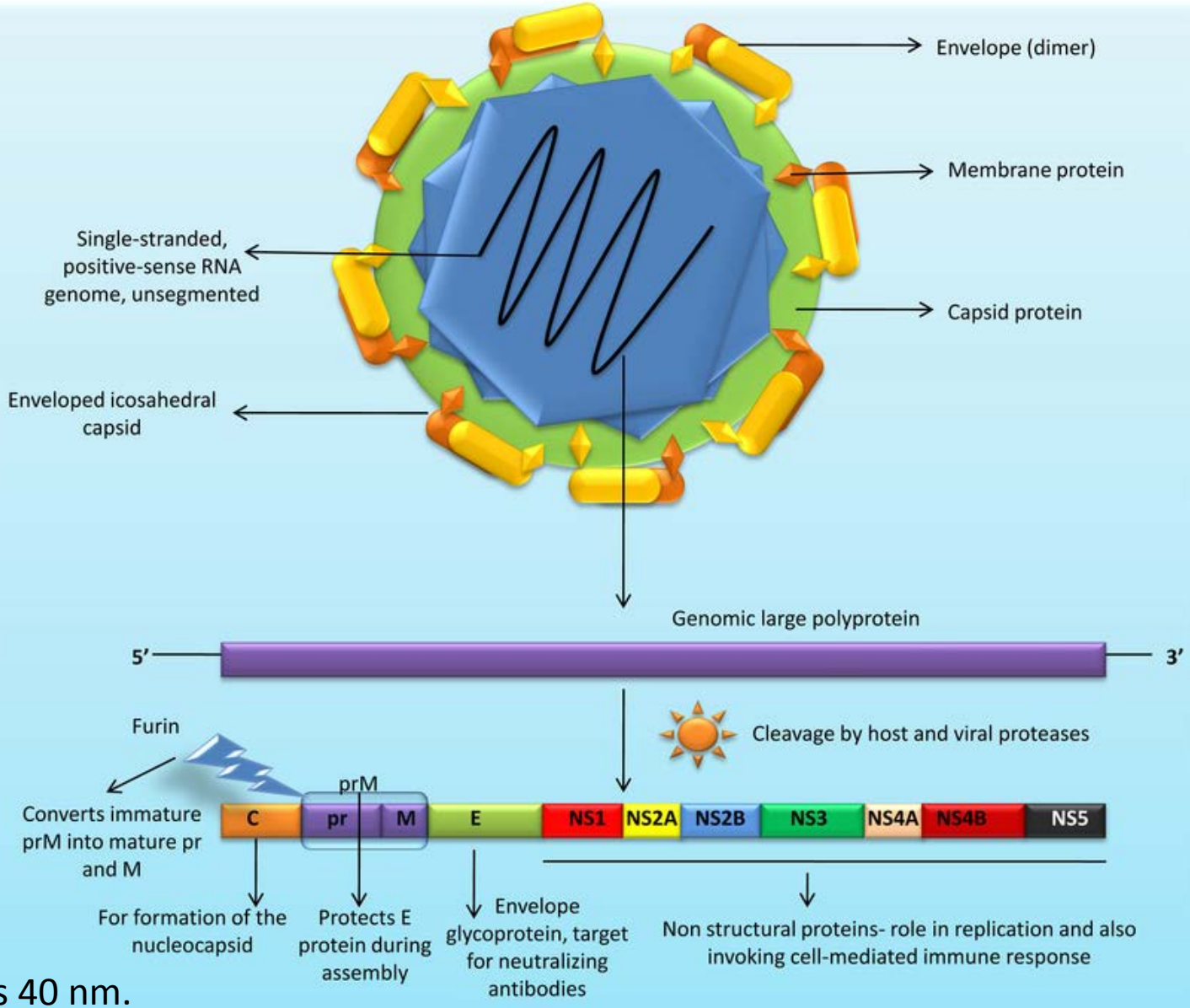
Presence of Zika virus RNA or antigen in serum or other samples (e.g. saliva, tissues, urine, whole blood); or

IgM antibody against Zika virus positive and PRNT₉₀ for Zika virus with titre ≥ 20 and Zika virus PRNT₉₀ titre ratio ≥ 4 compared to other flaviviruses; and
exclusion of other flaviviruses



Laboratory diagnosis of zika virus infection

- Genome detection by RT-PCR
- Virus isolation
- Serology
- Antigen detection by immunohistochemistry in the infected fetus
- Genome detection and plaque reduction neutralization are the gold standard methods.



Particle size is 40 nm.

https://www.researchgate.net/profile/Kuldeep_Dhama/publication/296700595/figure/download/fig3/AS:360408549478403@1462939502612/Figure-1-Structure-of-Zika-virus-and-its-genome-Zika-possess-ssve-sense-RNA-which-has.png

Genome detection



Based on 2 gene targets

- Pan-flavivirus specific:

NS5, prM, NS1, NS2b

- Zika virus specific: E region

- Multiplex RT-PCR for Zika, dengue and chikungunya viruses



Sources of Zika virus

- Serum/whole blood
- Urine
- CSF

- Saliva
- Semen
- Vaginal discharge
- Amniotic fluid
- Placenta and fetal tissues



Congenital infection

Zika virus RNA was detected in

- amniotic fluid of mothers
- tissue of fetuses

Primers and probes for Zika virus detection by real time RT-PCR

Primer and probe sets	Target gene	Primer/probe name	Sequence (5'-3')	Position
CDC1 (Lanciottii)	PrM	ZIKV 835	TTGGTCATGATACTGCTGATTGC	835–857
		ZIKV 911c	CCTTCCACAAAGTCCCTATTGC	911–890
		ZIKV 860-FAM	CGGCATACAGCATCAGGTGCATAGGAG	860–886
	E	ZIKV 1086	CCGCTGCCCAACACAAG	1086–1102
		ZIKV 1162c	CCACTAACGTTCTTTTGCAGACAT	1162–1139
		ZIKV 1107-FAM	AGCCTACCTTGACAAGCAGTCAGACAC TCAA	1107–1137
CDC2 (PAHO)	NS2b	Zika 4481	CTGTGGCATGAACCCAATAG	4434–4453
		Zika 4552c	ATCCCATAGAGCACCCTCC	4524–4505
		Zika 4507c-FAM	CCACGCTCCAGCTGCAAAGG	4479–4460

เปรียบเทียบผลประเมินการตรวจสอบพันธุกรรมไวรัสซิกา โดยวิธี RT-PCR รวม 4 วิธี

Virus Concentration (FFU)	Zika isolated No. 10/15			
	ผลตรวจ (average Ct value, duplicated)			Nested RT-PCR
	CDC1 (Lanciotti)	CDC2 (PAHO)	RealStar [®] (Altona)	
Zika 10 ^{*-3}	ไม่พบเชื้อ	ไม่พบเชื้อ	ไม่พบเชื้อ	ไม่พบเชื้อ
Zika 10 ^{*-2}	ไม่พบเชื้อ	ไม่พบเชื้อ	<u>พบเชื้อ(37.15)</u>	ไม่พบเชื้อ
Zika 10 ^{*-1}	ไม่พบเชื้อ	<u>พบเชื้อ(36.04)</u>	พบเชื้อ(34.27)	<u>พบเชื้อ</u>
Zika 10 ^{*0}	<u>พบเชื้อ (36.23)</u>	พบเชื้อ(33.61)	พบเชื้อ(31.95)	พบเชื้อ
Zika 10 ^{*1}	พบเชื้อ(33.00)	พบเชื้อ(29.49)	พบเชื้อ(29.16)	พบเชื้อ
Zika 10 ^{*2}	พบเชื้อ(29.96)	พบเชื้อ(26.24)	พบเชื้อ(25.95)	พบเชื้อ
Zika 10 ^{*3}	พบเชื้อ(26.51)	พบเชื้อ(22.91)	พบเชื้อ(22.71)	พบเชื้อ
Zika 10 ^{*4}	พบเชื้อ(22.96)	พบเชื้อ(19.47)	พบเชื้อ(19.06)	พบเชื้อ

เปรียบเทียบผลประเมินการตรวจสารพันธุกรรมไวรัสซิกา โดยวิธี RT-PCR รวม 4 วิธี

Virus Concentration (FFU)	Zika isolate No. 217/14			
	ผลตรวจ (average Ct value, duplicated)			Nested RT-PCR
	CDC1 (Lanciotti)	CDC2 (PAHO)	RealStar® (Altona)	
Zika 10 ^{*-3}	ไม่พบเชื้อ	ไม่พบเชื้อ	ไม่พบเชื้อ	ไม่พบเชื้อ
Zika 10 ^{*-2}	ไม่พบเชื้อ	ไม่พบเชื้อ	<u>พบเชื้อ(38.23)</u>	<u>พบเชื้อ</u>
Zika 10 ^{*-1}	ไม่พบเชื้อ	<u>พบเชื้อ(36.46)</u>	พบเชื้อ(34.76)	พบเชื้อ
Zika 10 ^{*0}	<u>พบเชื้อ(35.60)</u>	พบเชื้อ(32.81)	พบเชื้อ(31.96)	พบเชื้อ
Zika 10 ^{*1}	พบเชื้อ(31.81)	พบเชื้อ(29.29)	พบเชื้อ(29.09)	พบเชื้อ
Zika 10 ^{*2}	พบเชื้อ(28.34)	พบเชื้อ(26.13)	พบเชื้อ(25.76)	พบเชื้อ
Zika 10 ^{*3}	พบเชื้อ(24.89)	พบเชื้อ(22.53)	(22.14)	พบเชื้อ
Zika 10 ^{*4}	พบเชื้อ(21.29)	พบเชื้อ(19.02)	(18.78)	พบเชื้อ

Table 5. Zika Virus (ZIKV) Diagnostic Assays With Emergency Use Authorization From the Food and Drug Administration (FDA)

Test Category	Assay	Approval
Molecular ^a	CDC Trioplex Real-time RT-PCR Assay	Approved for Zika, chikungunya, and dengue testing on serum and CSF Additionally approved for Zika testing on whole blood and amniotic fluid
	Zika Virus RNA Qualitative Real-time RT-PCR (Focus Diagnostics, Cypress, California)	Approved for Zika testing on serum
	RealStar Zika virus RT-PCR kit US (Altona Diagnostics GmbH, Hamburg, Germany)	Approved for Zika testing on serum and urine
	Aptima Zika Virus Assay (Hologic Inc, Marlborough, Massachusetts)	Approved for Zika testing on serum and plasma
	Zika Virus Real-time RT-PCR test (Viracor-IBT, Lee's Summit, Missouri)	Approved for Zika testing on serum, plasma, and urine
	VERSANT Zika RNA 1.0 Assay (kPCR) Kit (Siemens Healthcare Diagnostics Inc, Newark, Delaware)	Approved for Zika testing on serum, plasma, and urine
	xMAP MultiFLEX Zika RNA Assay (Luminex Corp, Austin, Texas)	Approved for Zika testing on serum, plasma, and urine
	LightMix Zika rRT-PCR Test (Roche Molecular Systems, Pleasanton, California)	Approved for Zika testing on serum and plasma
Serology	CDC Zika MAC-ELISA for the detection of IgM	Approved for Zika testing on serum and CSF
	ZIKV Detect IgM Capture ELISA (InBiOS, Seattle, Washington)	Approved for Zika testing on serum

Abbreviations: CDC, Centers for Disease Control and Prevention; CSF, cerebrospinal fluid; Ig, immunoglobulin; MAC-ELISA, IgM antibody capture enzyme-linked immunosorbent assay; rRT-PCR, real-time reverse transcription–polymerase chain reaction; RT-PCR, reverse transcription–polymerase chain reaction.



Duration of genome detection

Serum: 3-5 d

Urine: 20 d

Saliva: 5-7 d

Semen: 2 - 6 mo.

Female genital secretion: 8 d



Biosafety issue on virus isolation

- Pregnant women should not handle virus isolation technique.
- BSL2 with BSL3 practice or BSL3 laboratory



Virus isolation

- *Toxorhynchites* mosquito (elephant mosquito or mosquito eater)
- C6/36
- Vero E6
- LLC-MK2
- Zika virus produces plaque in Vero cells.



http://farm9.static.flickr.com/8319/7922750140_68e2a305e4.jpg

Why it is difficult to isolate Zika virus?



Patients infected with Zika virus had low level of viremia (0.9×10^3 – 7.2×10^5 cDNA copies/ml); while it was 10^7 for chikungunya and 10^8 for DENV-2.



Antibody detection

Serology is of less value due to cross reaction among flaviviruses, especially dengue virus.

Serological techniques used:

ELISA IgM

Plaque reduction neutralization

Mouse neutralization

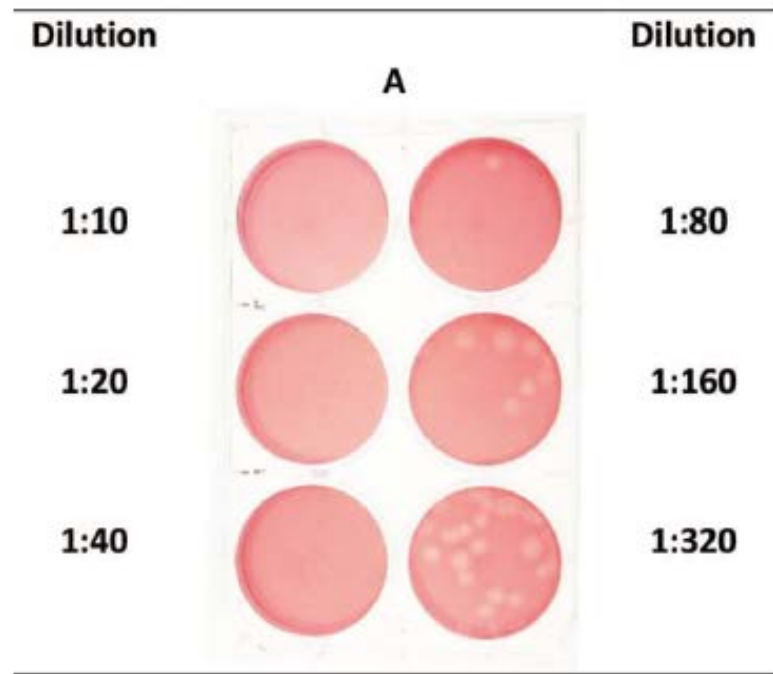
Hemagglutination-inhibition

Complement-fixation

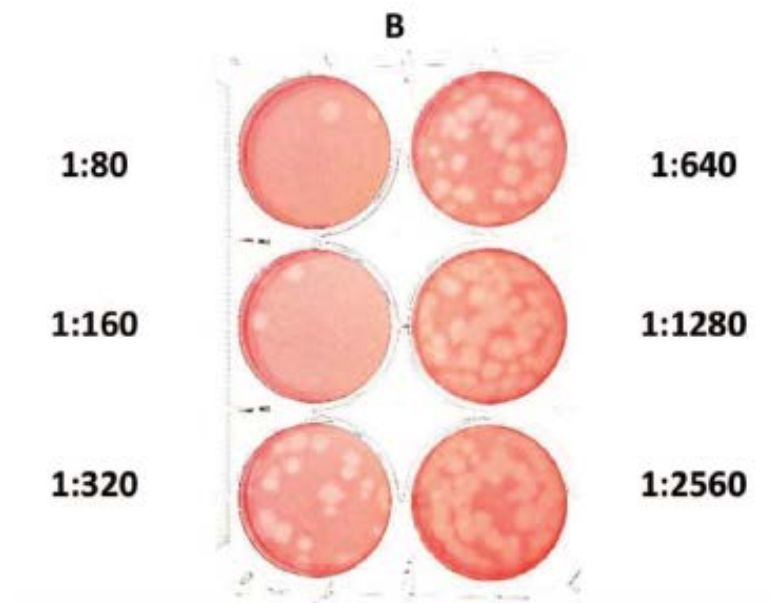


Zika patients may be positive in dengue IgM detection.

Serum from Zika patient may react with dengue NS1 Ag.



Plaque reduction neutralization test of acute (A) and convalescent (B) patient sera against Zika virus (ZIKV). Serial dilutions of patient serum were each incubated with 100 plaque-forming units of ZIKV for 1 hour at room temperature, then the serum-virus mixtures were inoculated onto Vero monolayers. Following an incubation of several days, cultures were fixed and stained, and virus-induced plaques were enumerated. Plaque counts lower than 10 were considered negative. In the examples shown here, the titers for both acute and convalescent sera are 1:160, indicating exposure to ZIKV, but the timing of exposure is inconclusive.



Confirmed case : A person with laboratory confirmation of recent Zika virus infection:

Presence of Zika virus RNA or antigen in serum or other samples (e.g. saliva, tissues, urine, whole blood); or

IgM antibody against Zika virus positive and PRNT₉₀ for Zika virus with titre ≥ 20 and Zika virus PRNT₉₀ titre ratio ≥ 4 compared to other flaviviruses; and exclusion of other flaviviruses

Table 2. Neutralization testing with heterologous flaviviruses of patients infected with ZIKV, Yap State, Micronesia, 2007*

Patient	Days after onset	PRNT ₅₀ titer									
		ZIKV	DENV1	DENV2	DENV3	DENV4	JEV	YFV	WNV	SLEV	MVEV
Primary flavivirus ZIKV											
822a	5	320	<10	<10	<10	<10	<10	<10	<10	<10	<10
822b	10	2,560	10	10	10	10	<10	<10	<10	<10	<10
Secondary flavivirus ZIKV (probable)											
817a	1	80	80	160	320	160	<10	<10	<10	40	40
817b	19	10,240	2,560	20,480	5,120	5,120	20	320	160	1,280	640
833a	1	160	320	80	40	20	<10	<10	<10	<10	<10
833b	19	81,920	20,480	5,120	5,120	1,280	<10	<10	80	320	320
844a	2	20	1,280	640	320	160	<10	<10	5	20	20
844b	16	10,240	40,980	10,240	5,120	1,280	5	<10	160	640	640

Doctrine of original antigenic sin

When Zika virus was detected in Thailand?

TABLE III. Proportion of adult indigenous residents of North Vietnam and Thailand possessing neutralizing antibodies against six arthropod-borne viruses.

Mouse NT

Virus	Number of LD ₅₀ used	Per cent. of sera positive from :		
		North Vietnam	Thailand	
		Tonkin	Bangkok	Chiangmai
		(50 sera tested)	(25 sera tested)	(25 sera tested)
JE	16	96	—*	—
	60	—	64	80
Ntaya	20	82	—	—
	80	—	84	64
Zika	80	—	8	0
	320	2	—	—
Ilhéus	60	—	4	0
	400	2	—	—
WN	250	—	36	16
Semliki	40	—	64	4
	65	22	—	—



Why there is no big Zika outbreak in Thailand and Southeast Asia?

- Cross protective immunity confers by dengue viruses???
- Dengue sera can neutralize Zika virus.
- Dengue sera mediates antibody dependent enhancement.
- Southeast Asian strain and South American strain is different in pathogenicity???



Why Zika virus outbreak emerged?

- NS1 codon usage adaptation to human could facilitate viral replication and increase viral titers.
- Control of *A. aegypti* (fed mostly on human blood) may let *A. albopictus* (fed on both human and animal blood) moved to the areas.

Why Zika virus outbreak emerged?



Warm climate and outbreaks

- With warm weather, mosquitoes fly more and bite more.
- Warm weather speeds up virus replication rate in mosquitoes.



Patients infected with Zika virus had low level of viremia (0.9×10^3 – 7.2×10^5 cDNA copies/ml); while it was 10^7 for chikungunya and 10^8 for DENV-2.



Zika patients may be positive in dengue IgM detection.

Serum from Zika patient may react with dengue NS1 Ag.

Safety guideline of blood transfusion

- For areas with no local transmission, it recommends donors who are at risk of Zika infection be deferred for 4 weeks.

People at risk : those who had symptoms consistent with Zika virus infection within the past 4 weeks, had sexual contact with someone who visited or lived in an area of local spread during the past 3 months, and anyone who traveled in the past 4 weeks to an area where the virus is circulating.

