Dipartimento di Sanità
Pubblica e Malattie Infettive

Sapienza
Università di Roma

CHIKUNGUNYA 2017: DATA, RESPONSE, ACTIONS AND CRITICAL ASPECTS

Rome, November 10, 2017

CHIK Outbreak in Italy: Virological and Diagnostic Issues

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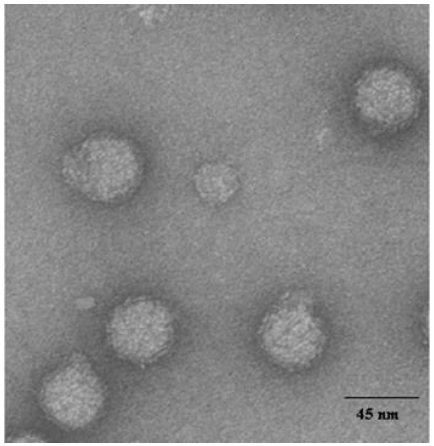
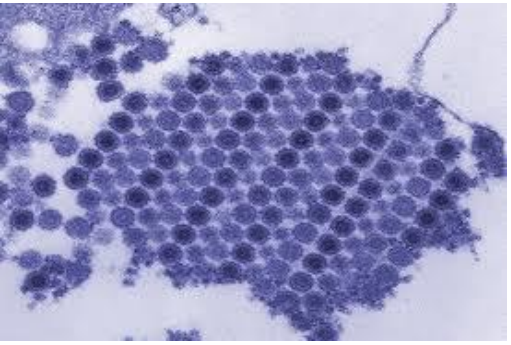
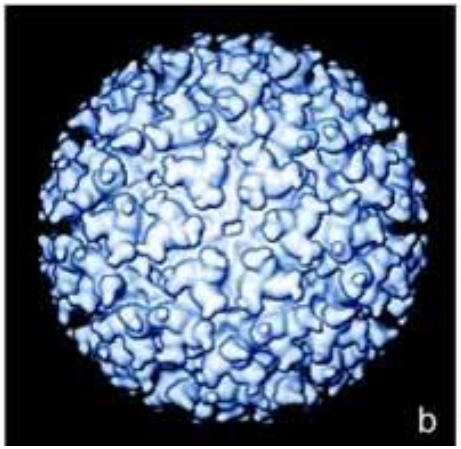
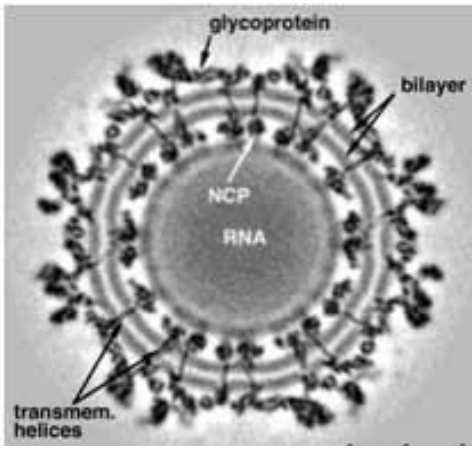
Chikungunya virus

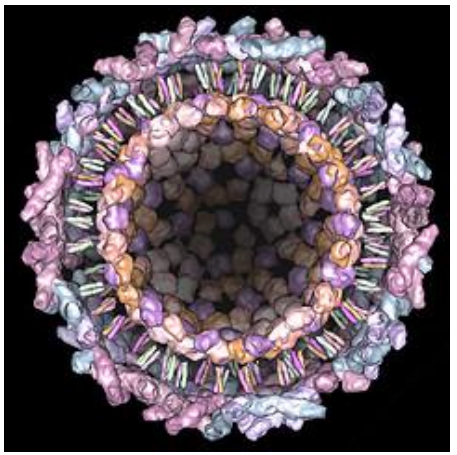




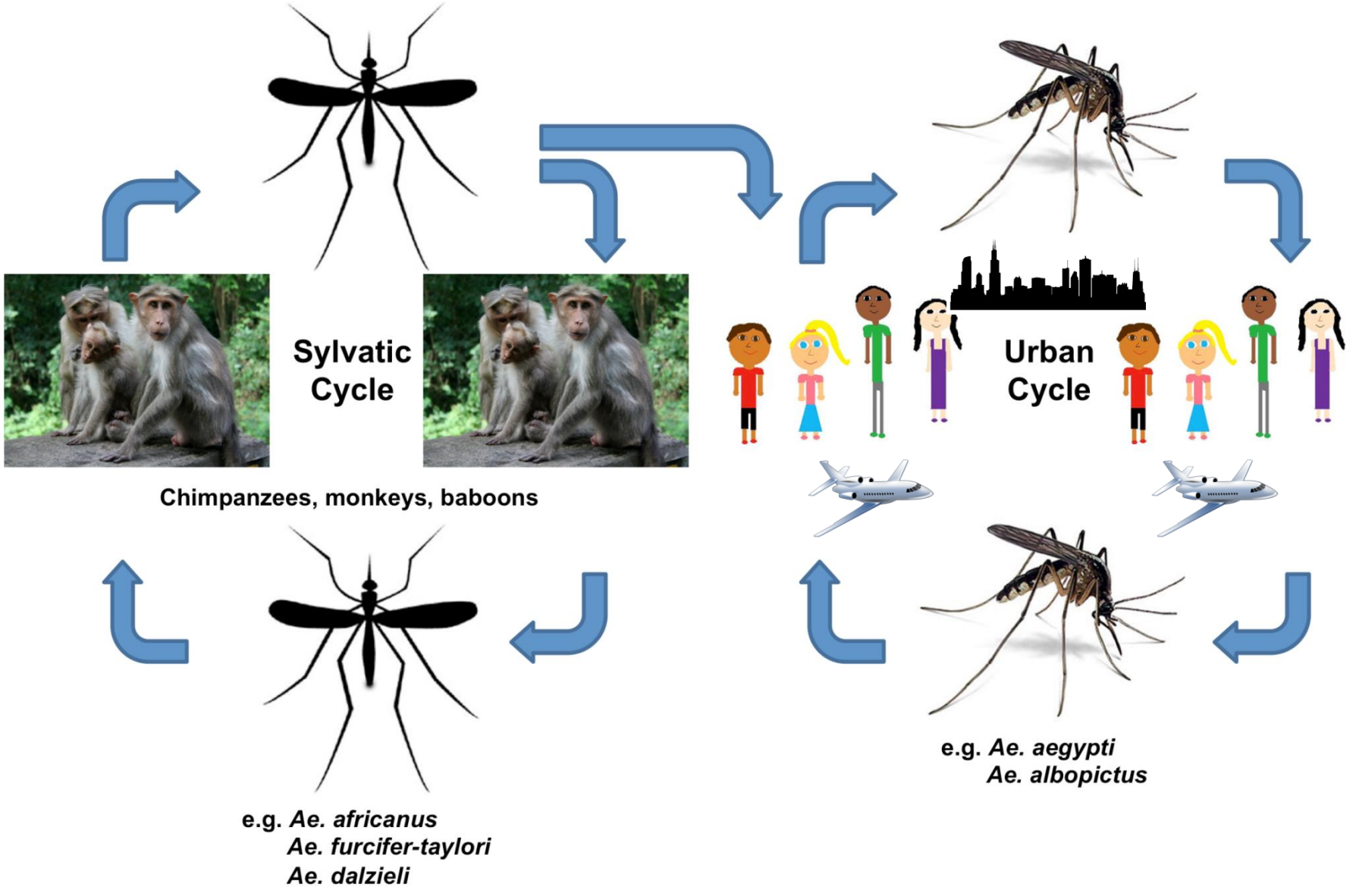
- CHIKV was first thought to be a strain of dengue virus (Ross, 1956)
- It's name comes from a Makonde (south Tanzania) word which can be roughly translated as "the disease that bends up the joints" (Ross, 1956)
- Family *Togaviridae*, genus alphavirus
- ~12Kb, single strand positive sense RNA genome
- Small and spherical (42nm), with *envelope* containing E1/E2/E3 glycoproteins (receptor function) and icosaedric capsid (C protein)
- Three major lineages: West African, ECSA, Asian-Caribbean





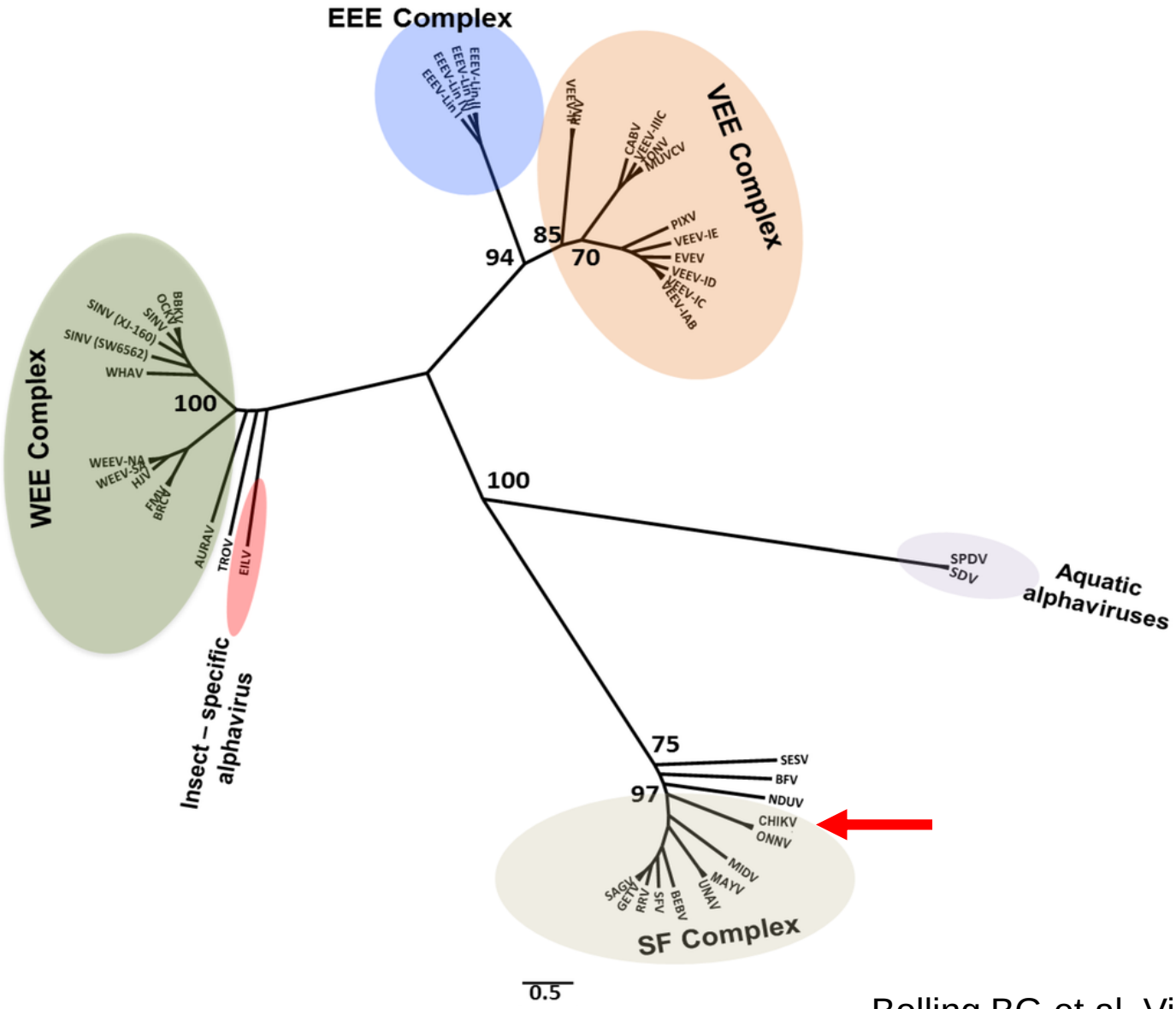


Enzootic and urban CHIKV transmission cycles



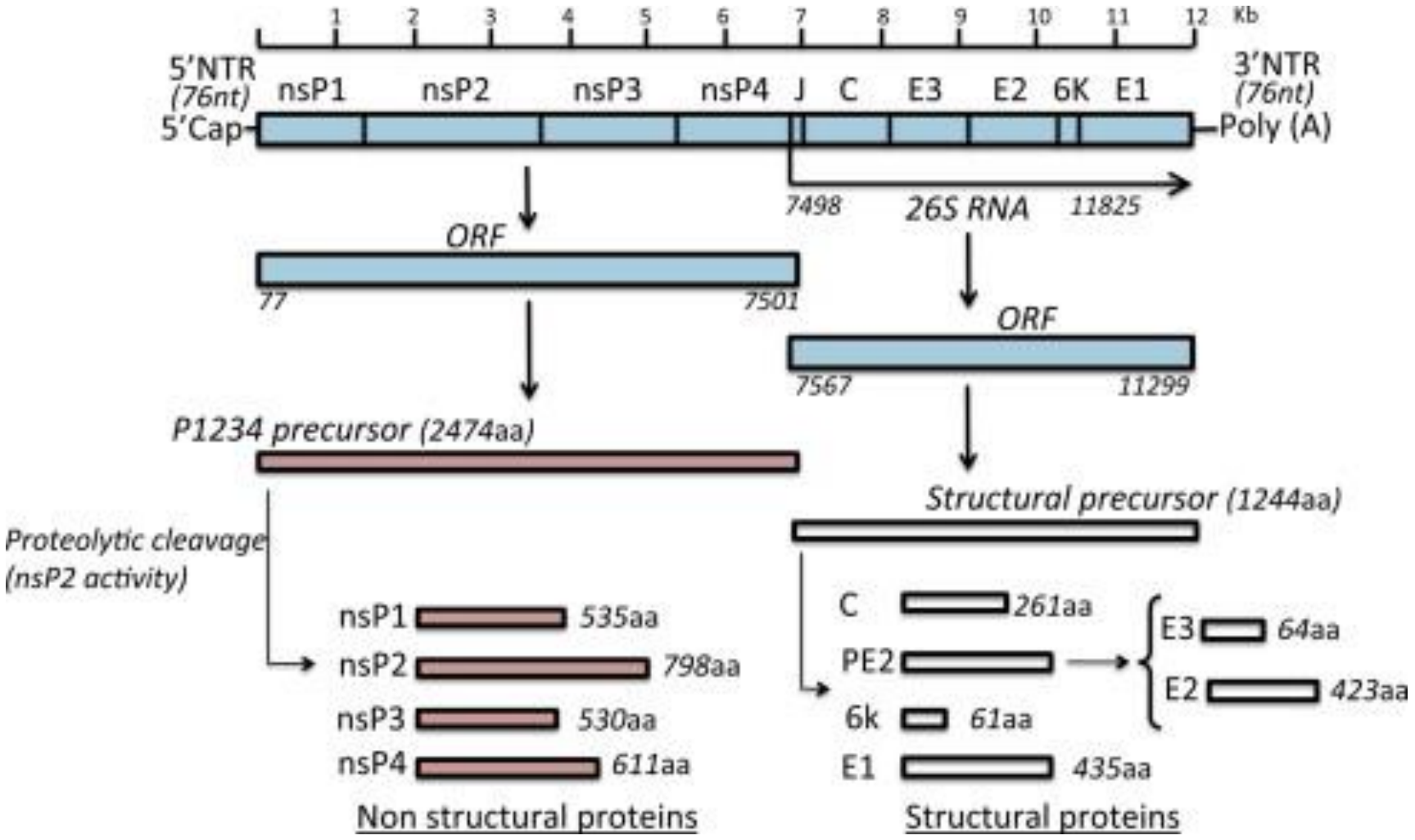
Thiboutot MM et al. PLoS Negl Trop Dis 2010

Unrooted phylogenetic tree of representative isolates of all alphavirus species generated from the E1 nucleotide sequences



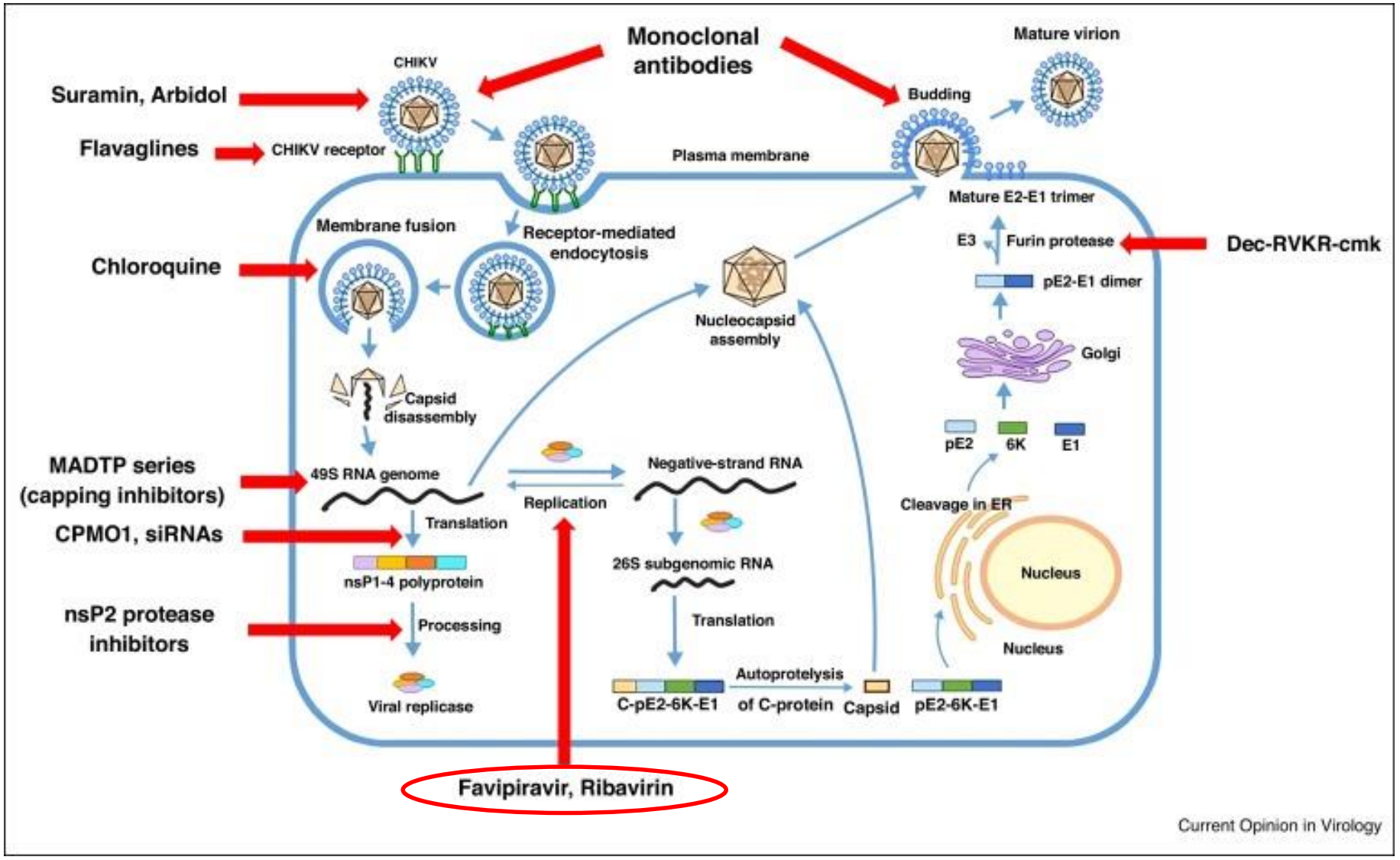
Bolling BG et al. Viruses 2015

CHIK genome organization and expression strategy



Thiberville SD, Antivir Res 2013

Every step of the viral life cycle can be targeted by antiviral molecules



Abdelnabi R, et al. Curr Opin Virol 2017

Chicungunya virus history

- The virus is believed to have originated in Africa, with subsequent spread to Asian countries probably occurring via shipping

- First outbreak was recognized during the modern scientific era in July, 1952 when an epidemic occurred in in present day Tanzania (Robinson 1955)

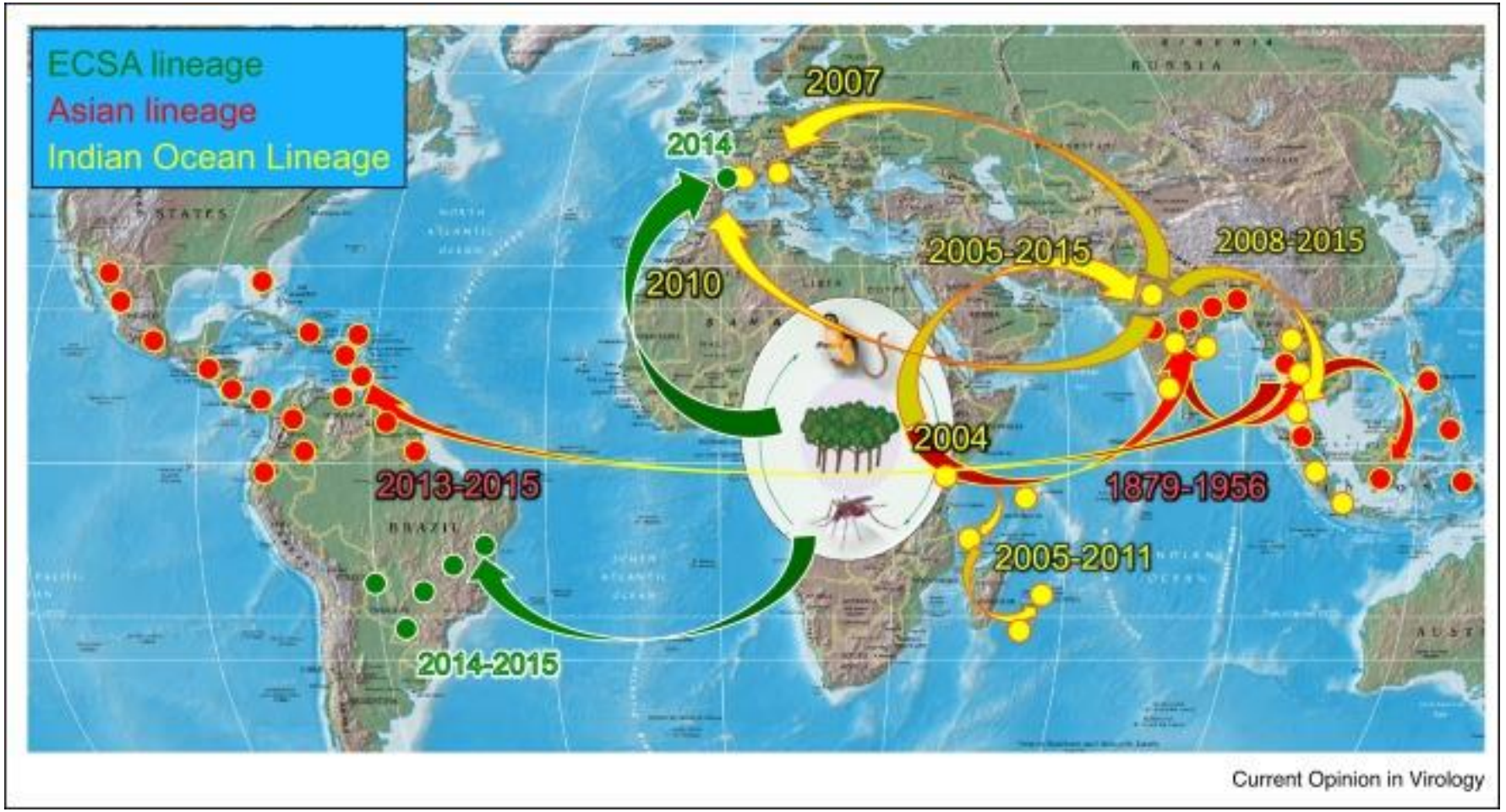
- The earliest confirmation of disease in Asia was reported from the Philippines in 1954. Outbreaks have subsequently been reported in southern and southeast Asia

- Since 1952, CHIKV has caused a number of epidemics, both in Africa and Southeast Asia, many of them having involved hundreds-of-thousands people

- In 2005 the largest Chikungunya fever epidemic on record occurred and rapidly spread to several countries in the Indian Ocean and India

- Since 2007, has spread widely, probably via viremic travelers, to initiate urban transmission in Europe, the South Pacific, and the Americas

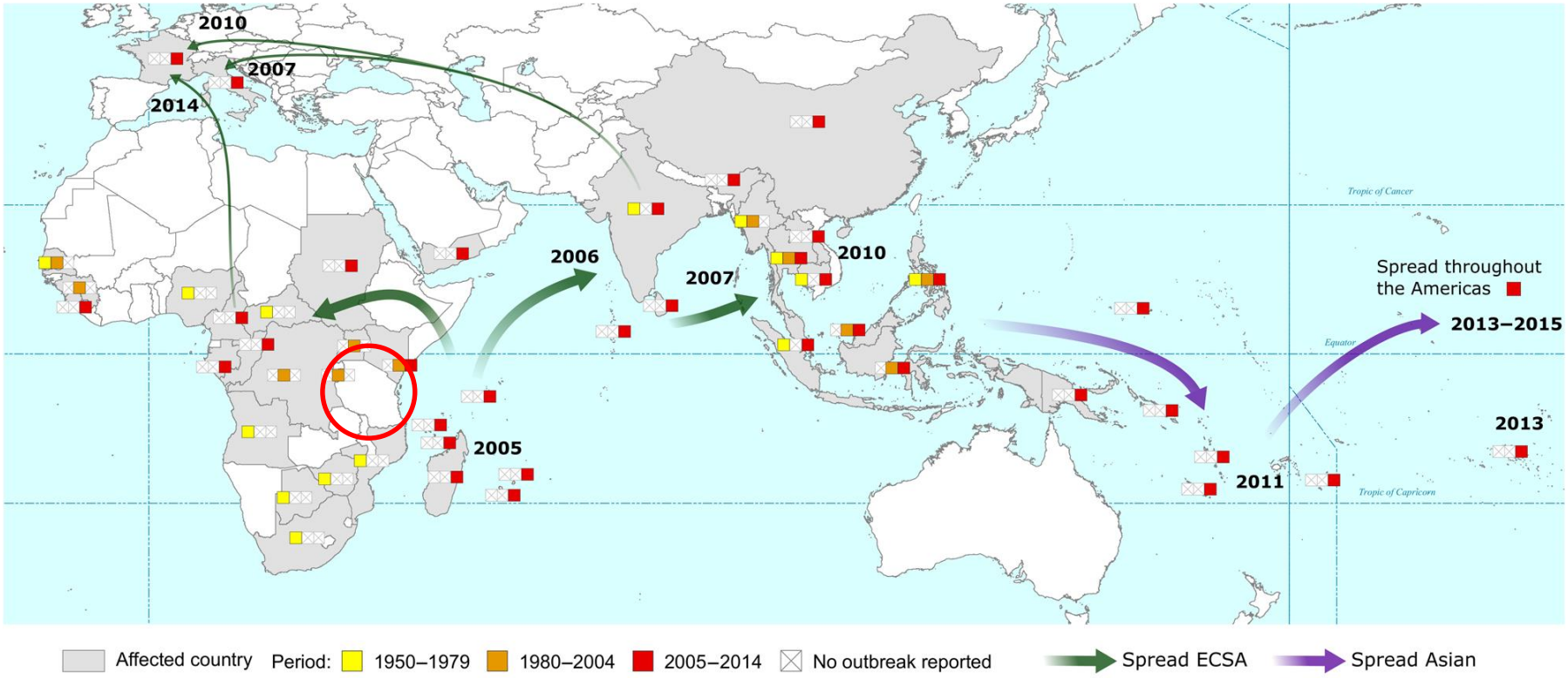
The historic spread of CHIKV from enzootic cycles in Africa to Asia, Europe and the Americas



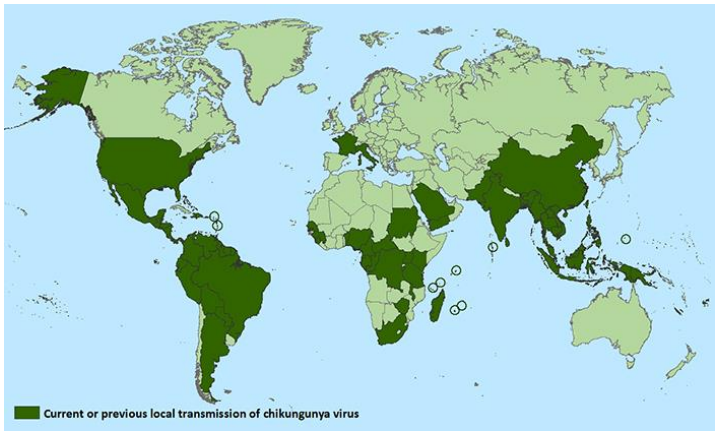
Tsetsarkin KA, Curr Opin Virol 2016

Chikungunya is on the move

How chikungunya went global in 10 years is a story of international travel, viral mutations and an accomplice with wings



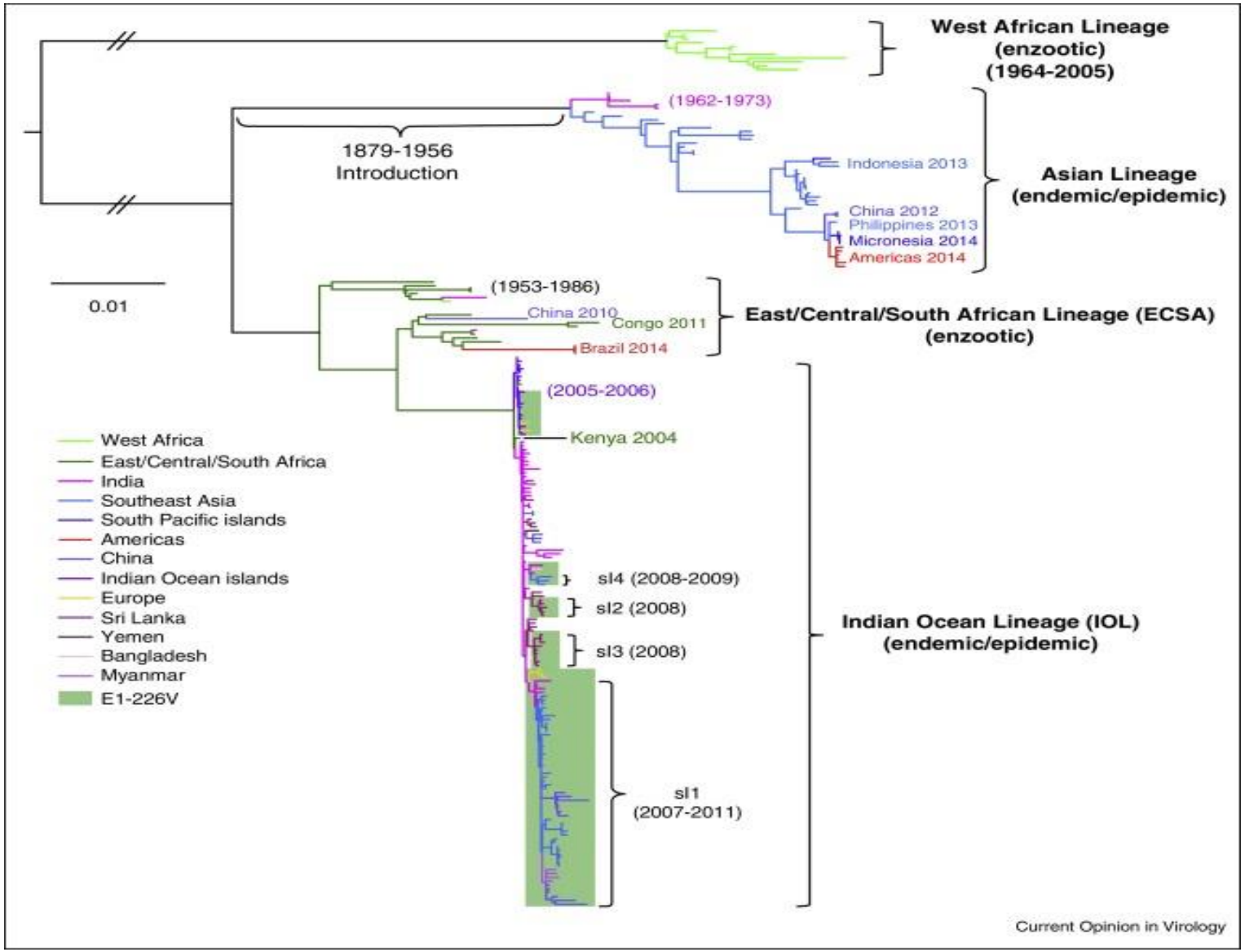
Zeller H, JID 2016



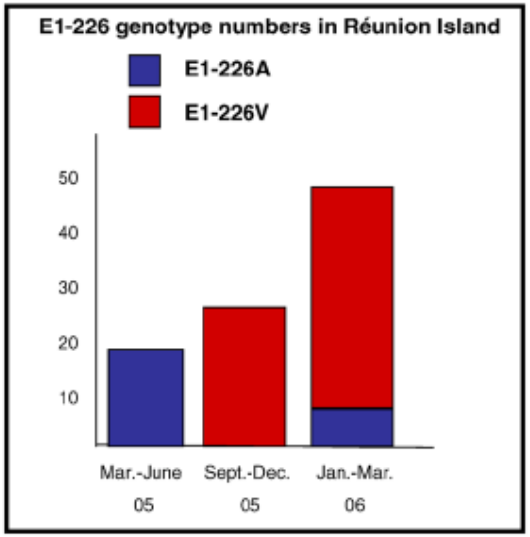
CDC

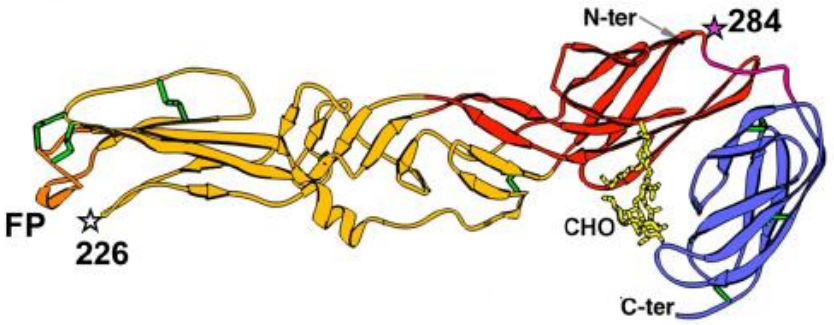
2016

Chikungunya lineages



Tsetsarkin KA, Curr Opin Virol 2016





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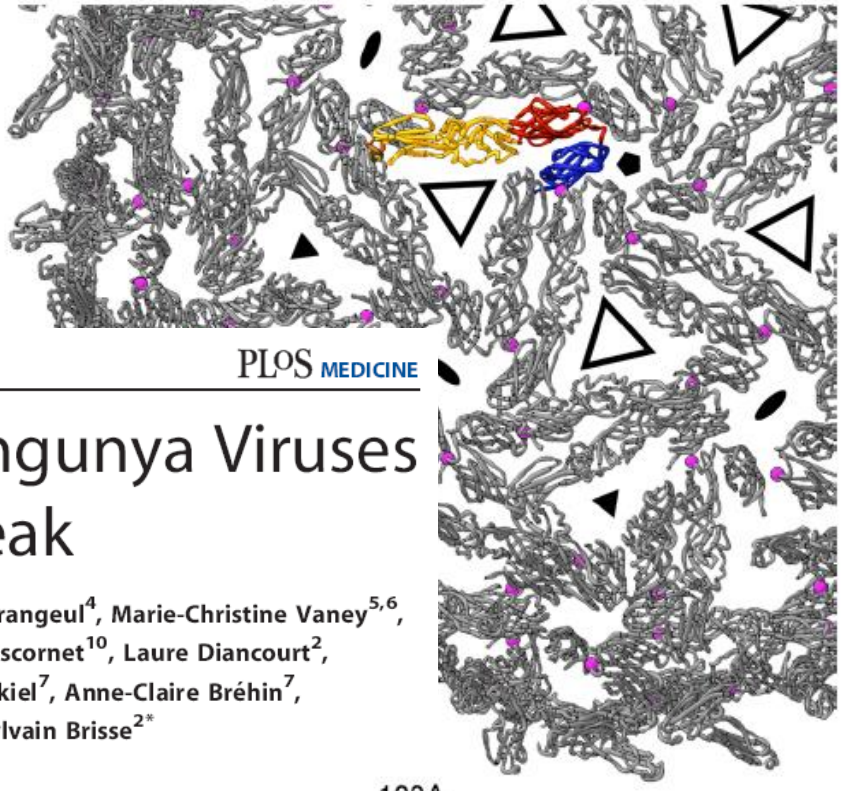


OPEN ACCESS Freely available online

PLOS MEDICINE

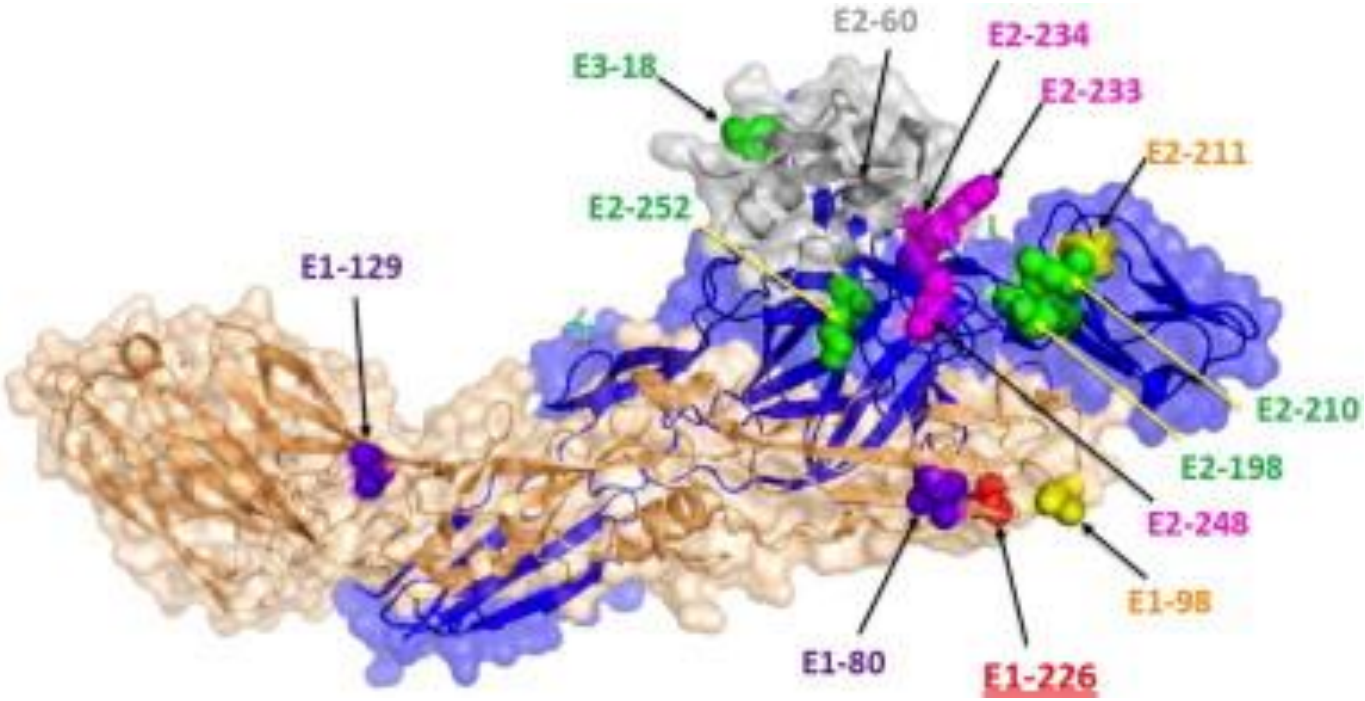
Genome Microevolution of Chikungunya Viruses Causing the Indian Ocean Outbreak

Isabelle Schuffenecker[1*], Isabelle Iteman[2], Alain Michault[3], Séverine Murri[1], Lionel Frangeul[4], Marie-Christine Vaney[5,6], Rachel Lavenir[2], Nathalie Pardigon[7], Jean-Marc Reynes[8], François Pettinelli[9], Leon Biscornet[10], Laure Diancourt[2], Stéphanie Michel[1], Stéphane Duquerroy[5,6,11], Ghislaine Guigon[2], Marie-Pascale Frenkiel[7], Anne-Claire Bréhin[7], Nadège Cubito[1], Philippe Desprès[7], Frank Kunst[12], Félix A. Rey[5,13], Hervé Zeller[1], Sylvain Brisse[2*]

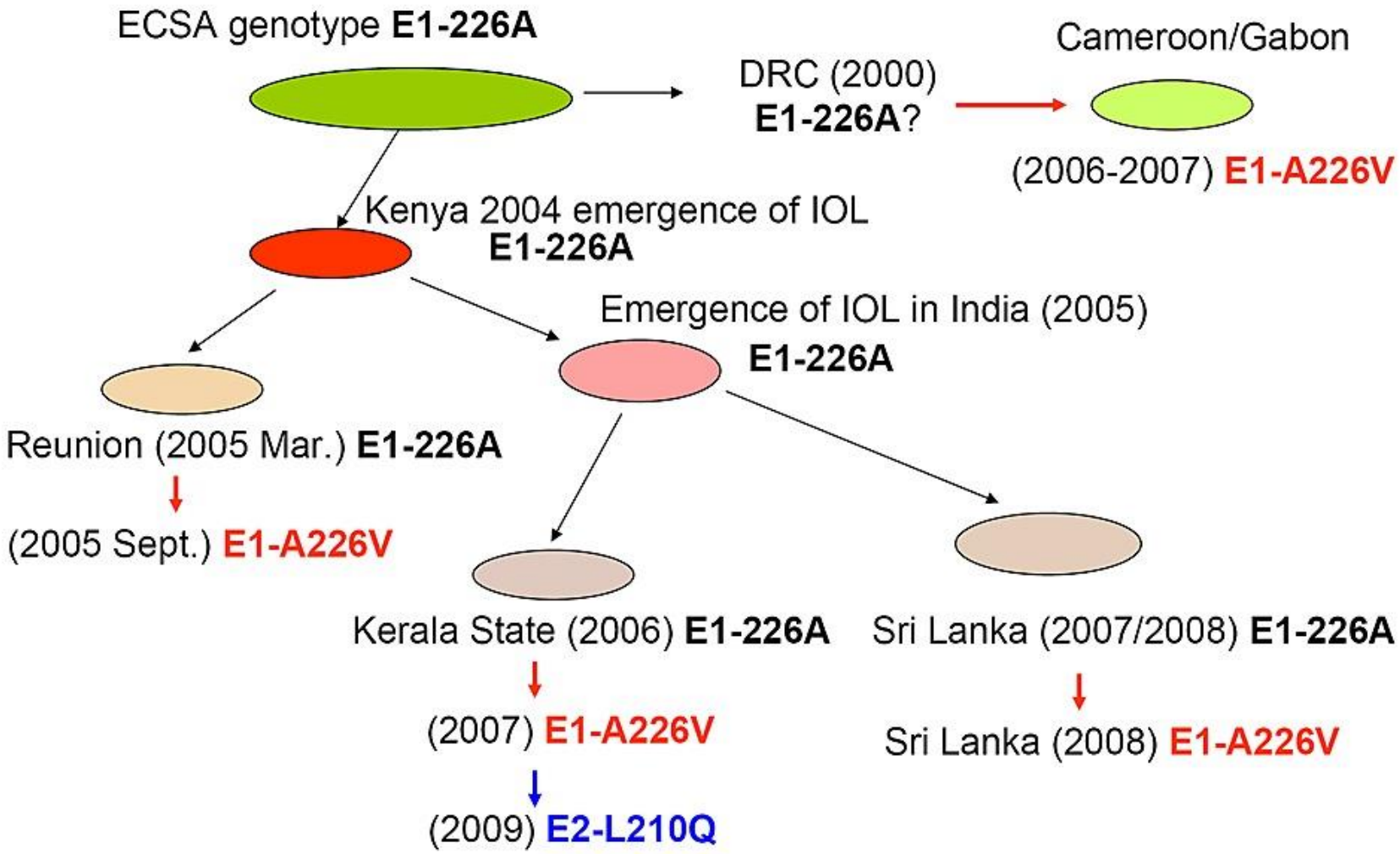
Figure 1. Localization of the E1 Changes on the 3D Structure Modelled from the Crystal Structure of SFV E1

Envelope glycoprotein substitutions that affect CHIKV fitness for transmission by *A. albopictus*

based on a 3D model of E3/E2/E1 spike



Weaver S.C. et al. Antiv Res 2015

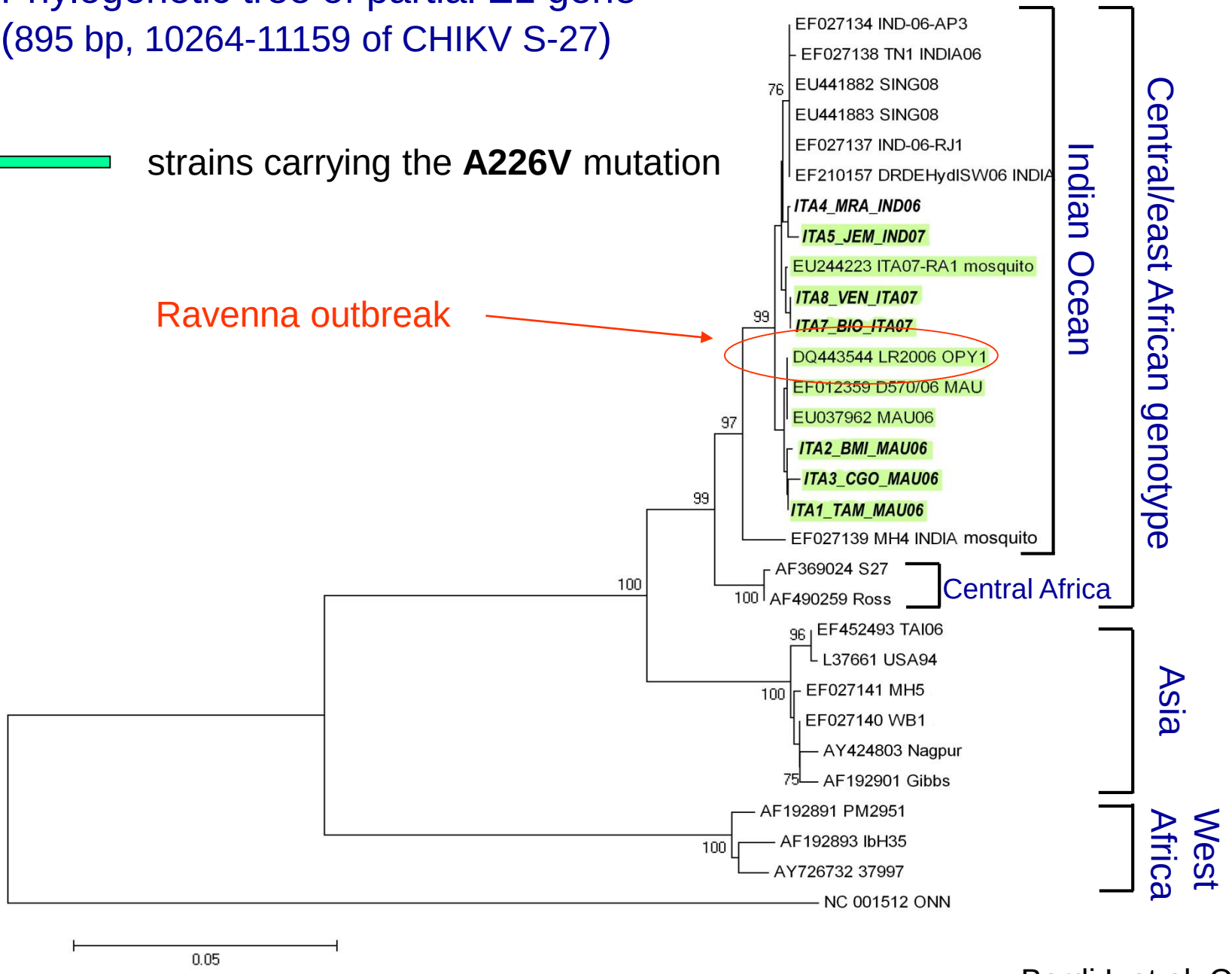


Evolutionary history of the E1-A226V and E2-L210Q substitutions in different CHIKV lineages of the ECSA clade. Black arrows correspond to the emergence and movement of the CHIKV lineages with the E1-226A residue. Red arrows correspond to the acquisition of the E1-A226V substitution. Blue arrow corresponds to acquisition of the E2-L210 substitution.

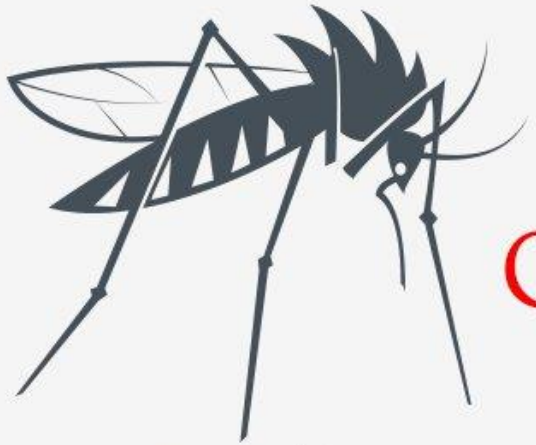
Tsetsarkin KA et al. Plos Pathogens 2007

Phylogenetic tree of partial E1 gene
(895 bp, 10264-11159 of CHIKV S-27)

strains carrying the **A226V** mutation



Bordi L et al. CID 2008

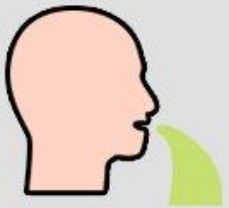


Symptoms of Chikungunya

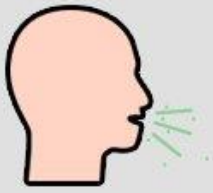
Rash

Back Pain

Joint Pain



Vomiting



Nausea

Headache

Chills

Fever

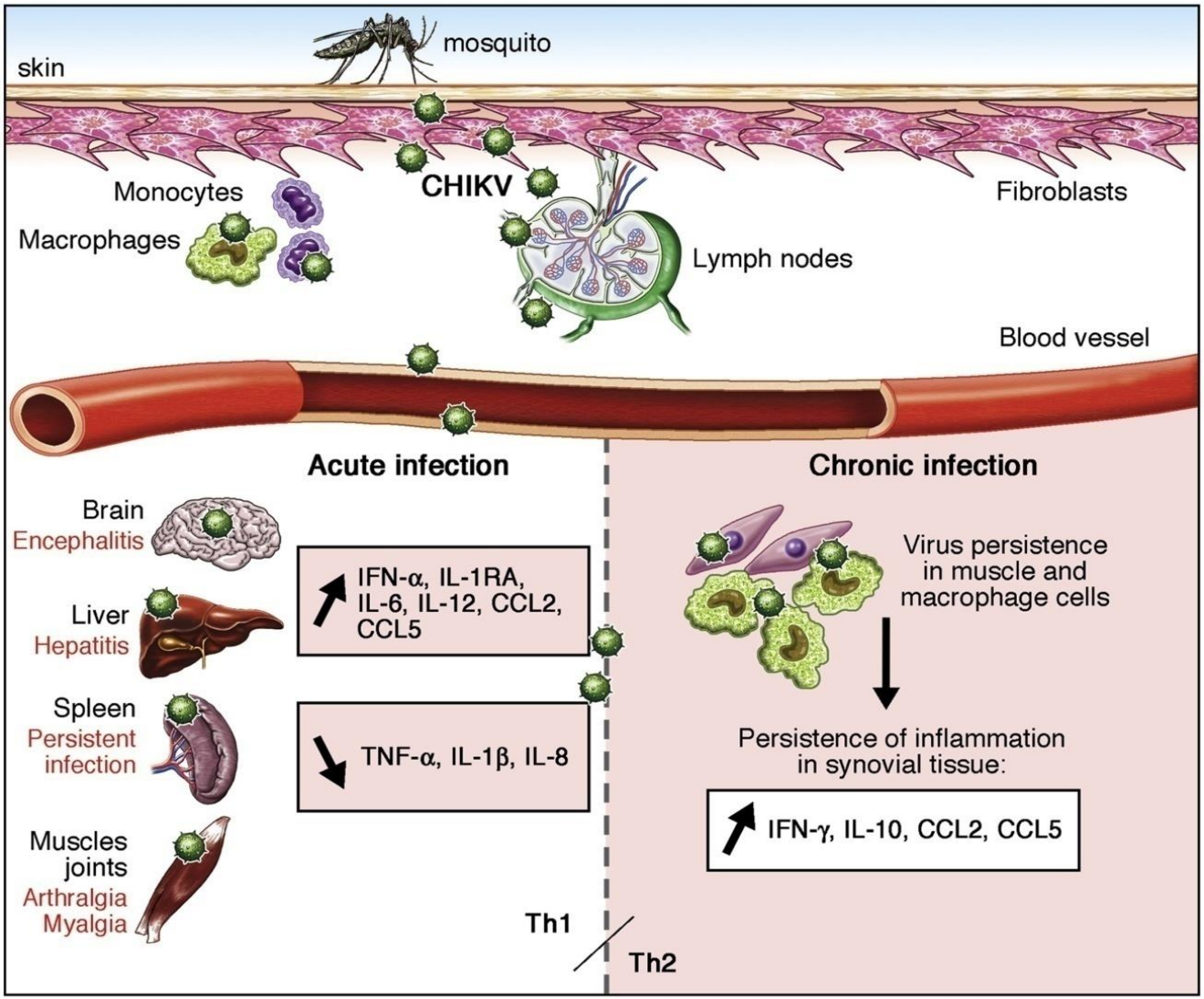
Stop The Hysteria,
Stop The Chikungunya!

Clinical similarities with dengue fever

Clinical Features	Chikungunya Virus (CHIKV)	Dengue Virus (DENV)
1) Fever, asthenia	Common	Common
2) Myalgia	Possible	Very common
3) Polyarthritis	Very Common, edematous	None
4) Tenosynovitis	Yes	None
5) Leukopenia	None	Yes
6) Thrombocytopaenia	None	Yes
7) Rash	Days 1–4, important skin edema	Days 3–7
8) Retro-orbital pain	Rare	Common
9) Hypotension	Possible	Common, Days 5–7
10) Minor bleeding	Chronic polyarthritis up to 1 year	Common
11) Second stage	Possible; Tenosynvovitis at M2–M3 Raynaud's syndrome at M2–M3	Fatigue up to 3 mo

Thiboutot MM et al. PLoS Negl Trop Dis 2010

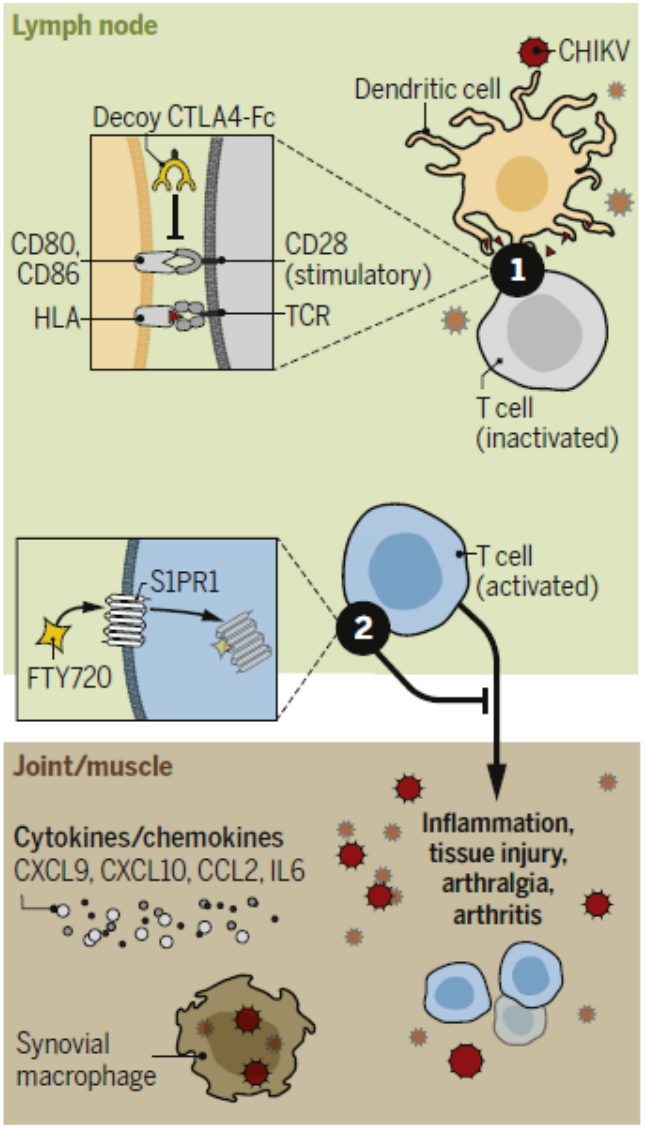
Chronic arthralgia for as long as 18 months after CHIKV infection is associated with the persistence of RNA in synovial tissue and with expression of IFN-α, IL-10, and CCL2 and proinflammatory cytokines



Petitdemange C, J Allerg Clin Immunol 2015

Sarah Crunkhorn
Nature Reviews Drug Discovery | Published online 30 Mar 2017

CHIKV-induced joint swelling seems to be an immune-mediated response involving CD4+ T cells



Mouse model

Treatment with abatacept (CTLA4–IgG, which blocks T cell activation) and a neutralizing Hu-Mab anti-CHIKV 3 days after CHIKV infection completely abolished foot swelling at day 7 (Miner J. J. *et al. Sci Transl Med 2017*)

Fingolimod (sphingosine 1-phosphate receptor modulator that blocks T cell egress from lymphoid organs) limited the migration of activated and CHIKV-specific CD4+ T cells into the virus-infected joints, which reduced the severity of joint vascular leakages, oedema in the subcutaneous region, and inflammation and necrosis of muscles (Teo T.-H. *et al. Sci Transl Med 2017)*

Gasque P et al. Sci Transl Med 2017

EU case definition. Laboratory Criteria

A. Probable case

- Detection of chikungunya specific IgM antibodies in a single serum sample

B. Confirmed case

- At least one of the following four:
  - ✓ Isolation of chikungunya virus from a clinical specimen
  - ✓ Detection of chikungunya viral nucleic acid from a clinical specimen
  - ✓ Detection of chikungunya specific IgM antibodies in a single serum sample AND confirmation by neutralisation
  - ✓ Seroconversion or four-fold antibody titre increase of chikungunya specific antibodies in paired serum samples

EVD-LabNet

Modified from:
EVD-LabNet Assessment laboratory preparedness and response to chikungunya virus, updated September 2017.

CHIKV Laboratory Diagnosis

Direct methods

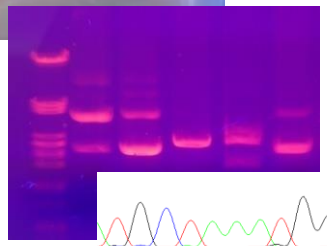
Various biological samples

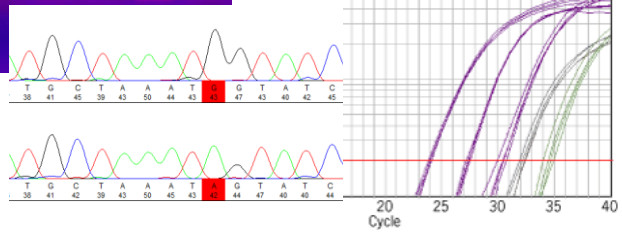
- **Direct detection**
- **Viral isolation in cell cultures**
- **RT-PCR (+Sequencing)**
- **Real-Time RT-PCR**

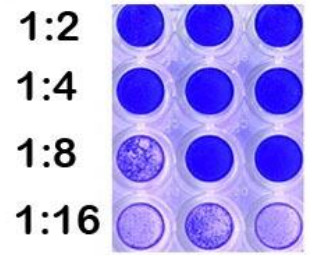
Indirect methods

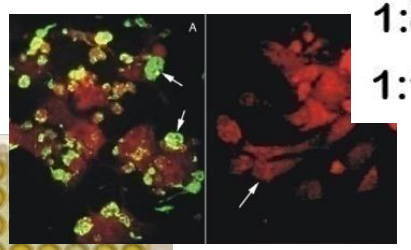
Serum/Plasma/Blood/other fluids

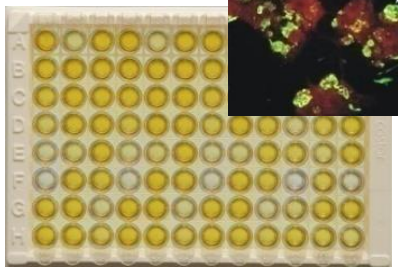
- **ELISA**
- **IFA**
- **Neutralization test**
- **T cell immunity**











Laboratory diagnosis of CHIK

- Challenges for the diagnosis or arboviral infections
  - Few commercial kits
  - Reagents and control samples rare
  - Cross-reactivity of Ab within same virus family

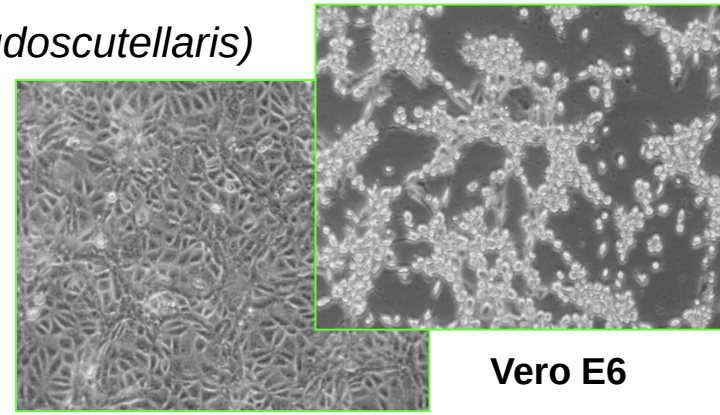
- Critical issues
  - Epidemiological link with provenance areas (for travelers) and alert systems to inform distant sytes
  - Endemic viruses surveillance in place
  - Risk perception by of physicians (and general pratictioners)
  - Availability of sequential sampling

Virus Isolation (in BSL3)

- **Time of collection after onset of symptom:** 1-7 days
- **Specimen:** serum, plasma, PBMC, autopsy tissues

  (liver, lung, lymph nodes, thymus, corneal swab, bone marrow)
- **Storage of specimen:** 4/8˚ C for 24h, -80˚ C for longer periods
- **Cell culture:**
  - mosquito cell lines C6/36 (*Ae. albopictus*), AP61 (*Ae. Pseudoscutellaris)*
  - mammalian cell lines (Vero, LLCMK2, BHK21)
- **Confirmation of viral: isolation** by IFA or PCR
- **Time to result:** 1-2 weeks

Since viremia is only short-lived, negative results from virus isolation should not be used to rule out CHIKV diagnosis.



Vero E6

Clinical specimens may also be inoculated by intracranial route in suckling mice or intrathoracic inoculation of mosquitos

Inhalation of aerosolised CHIKV in a laboratory setting may lead to CHIKV infection

Microneutralization ASSAY

2-fold dilutions

Add Sera

Add Virus

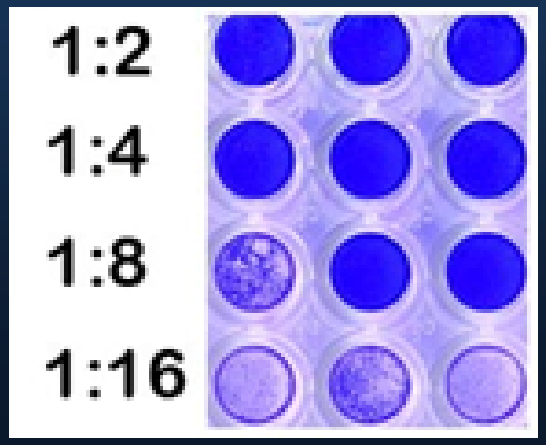
100 $TCID_{50}$/well

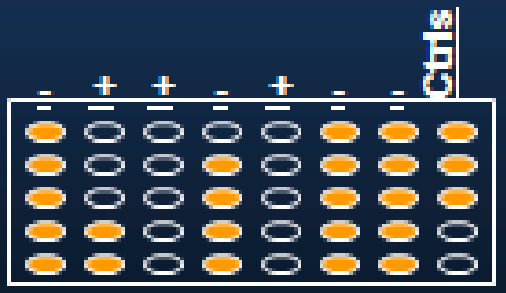
1-2H 37C

Add susceptible Cells

18H 37C

CPE





Unsperger et al. Emerg Infect Dis. 2009

Molecular diagnosis of CHIK

- Various RT-PCR protocols have been published

- Various commercial CHIKV RT-PCR kits are available (CE/IVD):
  - ✓ Altona - RealStar® Chikungunya RT-PCR Kit 2.0 (5 cp/reaction)
  - ✓ RAS Lifesciences Amplisure® Chikungunya RT PCR Kit
  - ✓ LiferiverTm Chikungunya Virus Real Time RT-PCR Kit
  - ✓ SentosaTM SA CHIKV RT-PCR Test
  - ✓ abTESTM CHIKU 1 qPCR Kit
  - ✓ Certest Biotec – Viasure Zika, Dengue, Chikungunya Real Time Detection Kit (>=10 cp/reaction)
  - ✓ Genome Diagnostics – Geno-Sen's CHIKV Real Time PCR
  - ✓ ELITECH – FTD Zika/Dengue/Chik (10^4 cp/ml)
  - ✓ BIONEER – Accupower ZIKV, DENV, CHIKV Multiplex Real-Time RT-PCR
  - ✓ *Roche-TibMolbiol LigthMix Chikungunya virus*

EVD-LabNet

Modified from:
EVD-LabNet Assessment laboratory preparedness and response to chikungunya virus, updated September 2017.

Am. J. Trop. Med. Hyg., 77(3), 2007, pp. 521–524

Short Report: Rapid Detection and Quantification of Chikungunya Virus by a One-Step Reverse Transcription–Polymerase Chain Reaction Real-Time Assay

Fabrizio Carletti, Licia Bordi, Roberta Chiappini, Giuseppe Ippolito, Maria R. Sciarrone, Maria R. Capobianchi, Antonino Di Caro,* and Concetta Castilletti

National Institute for Infectious Diseases L. Spallanzani, Rome, Italy

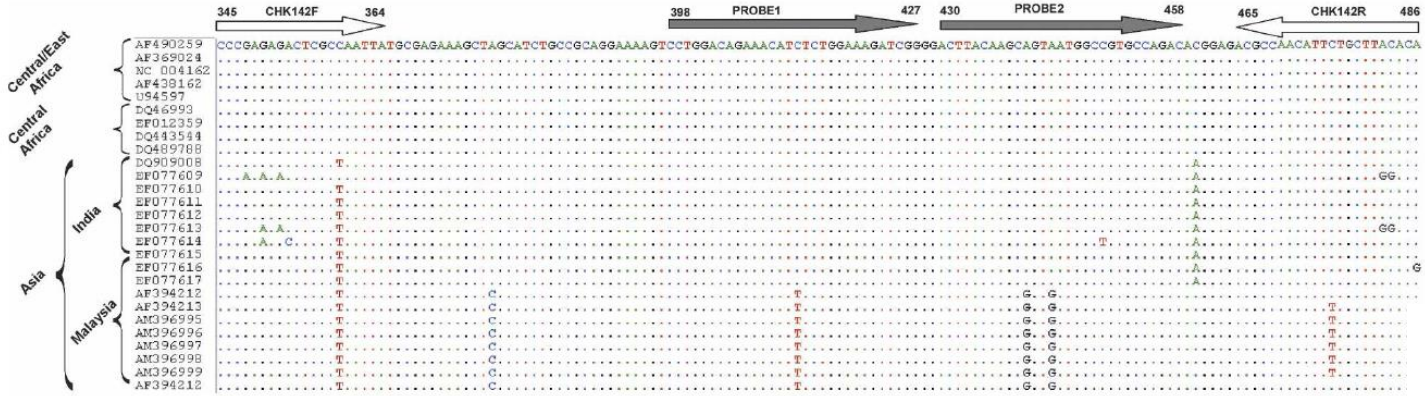
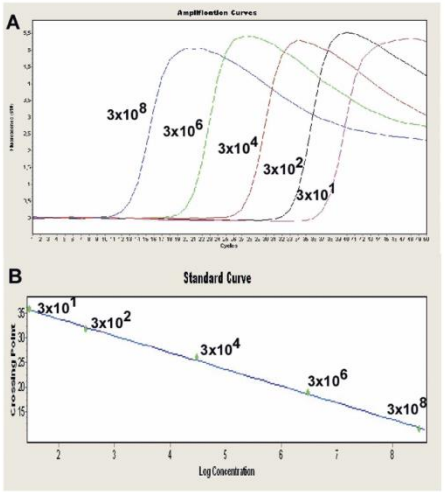
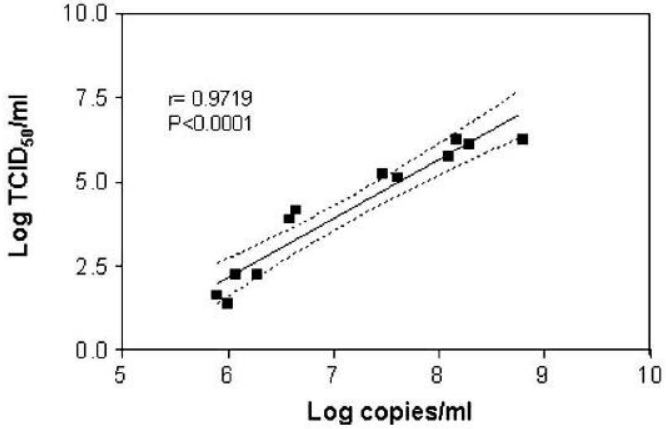


FIGURE 1. Multiple alignment of the nonstructural protein 1 regions of Chikungunya virus (CHIKV) with respect to the reference strain GenBank accession numbers of representative sequences are listed on the left (Ross reference strain accession no. AF490259). The CHIKV strains used for sequence alignments belong to clusters from Central Africa, Central/East Africa, and Asia. Dots indicate sequence identity. The figure appears in color at www.ajtmh.org.





Application of CHIKV qPCR to clinical samples

Viremia was detected during the acute phase; viral load ranged from $1.3x10^5$ to $5.9x10^8$ cp/ml

Patient ID, gender , age	Travel history, beginning of symptoms	Sampling date	RT-PCR (nsP1 /E1)	qPCR (cp/ml)	Viral Isolation (strain designation)	Ab IFA IgG (titers)	Ab IFA IgM
CG L, M, 48	Mauritius 27/03/06	31/03/06	Pos	1.3×10^5	Neg	Neg***	Pos§
		28/04/06	nd*	Neg**	nd	≥1:320	Pos
TAM, M, 45	Mauritius 03/04/06	05/04/06	Pos	1.3×10^8	Pos (CHKV ITA1 TAM)	Neg	Neg***
		27/04/06	Neg	Neg	nd	≥1:320	Pos
RS, M, 56	Seychelles 30/05/06	06/06/06	Pos	2.2×10^7	nd	Neg	Neg
		10/10/06	nd	Neg	nd	1:320	Neg
MR, F, 35	India 07/09/06	09/09/06	Pos	5.9×10^8	Pos (CHKV ITA3 MR)	Neg	Neg
		29/09/06	nd	nd	nd	1:320	Pos

*nd: not done; ** neg:<$4x10^3$ cp/m; *** neg:<1:20; § pos:>1:20 (titre if available)

Carletti er al. Am J Trop Med Hyg 2007

Molecular diagnosis of CHIK

- Various RT-PCR protocols have been published

- Various commercial CHIKV RT-PCR kits are available (CE/IVD):
  - ✓ Altona - RealStar® Chikungunya RT-PCR Kit 2.0 (5 cp/reaction)
  - ✓ RAS Lifesciences Amplisure® Chikungunya RT PCR Kit
  - ✓ LiferiverTm Chikungunya Virus Real Time RT-PCR Kit
  - ✓ SentosaTM SA CHIKV RT-PCR Test
  - ✓ abTESTM CHIKU 1 qPCR Kit
  - ✓ Certest Biotec – Viasure Zika, Dengue, Chikungunya Real Time Detection Kit (>=10 cp/reaction)
  - ✓ Genome Diagnostics – Geno-Sen's CHIKV Real Time PCR
  - ✓ ELITECH – FTD Zika/Dengue/Chik (10^4 cp/ml)
  - ✓ BIONEER – Accupower ZIKV, DENV, CHIKV Multiplex Real-Time RT-PCR
  - ✓ Roche-TibMolbiol LigthMix Chikungunya virus

EVD-LabNet

Modified from:
EVD-LabNet Assessment laboratory preparedness and response to chikungunya virus, updated September 2017.

Serological diagnosis of CHIK

- IFA, ELISA, virus neutralization, HA-inhibition for CHIKV Ab
- VNT, PRNT require BSL3 practices and containment equipment
- ELISA is the most commonly used method. Examples of commercial kits:
  - ✓ Genway CHIKV IgG capture ELISA.
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  - ✓ Abcam IgM sandwich IgM/IgG ELISA.
  - ✓ Creative diagnostics IgM or IgG capture ELISA.
  - ✓ IBL International, Hamburg, Germany Chikungunya IgM. and IgG--capture ELISA
  - ✓ Euroimmun, Lübeck, Germany Anti-Chikungunya Virus ELISA IgM and IgG
  - ✓ Focus Diagnostics - Chikungunya virus IgM e IgG IFA ed ELISA Kit
  - ✓ ARUP Laboratories - Chikungunya virus IgM e IgG ELISA Kit
- Very few reports on the systematic and comparative evaluation of these commercial products. Available data show wide range of sensitivity and specificity:
  - ✓ Sensitivity range: 79-85% (IgM) and 52-88% (IgG)
  - ✓ Specificity range: 82-88% (IgM) and 95-96% (IgG)
- Indirect Immunofluorescence-based tests (IFA) are commercially available:
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  - ✓Euroimmun, Lübeck, Germany IFA.
- A few commercial RDT are available, but performed poorly
  - ✓Duo Dengue IgG/IgM-CHIK IgM Rapid Test SD Bioline Chikungunya
  - ✓OnSite Chikungunya IgM Combo Rapid TestIgM

EVD-LabNet

Modified from: EVD-LabNet Assessment laboratory preparedness and response to ChikV, updated September 2017.

Serological diagnosis: General recommendations

- Confirmatory testing is crucial
- Paired serum samples collected 2.3 weeks apart allow establishment of seroconversion or significant (≥4fold) increase of Ab titer
- Information required for adequate result interpretation:
  - ✓ provenance
  - ✓ start of symptoms
  - ✓ previous arbovirus infections
  - ✓ arbovirus vaccination history
- Complementary health status information
  - ✓ Pregnancy
  - ✓ Immunodeficiency
  - ✓ ……

Laboratory diagnosis of CHIK

Recommended algorithm for returning travelers and for newly affected regions

- **Sample taken ≤ 7 days post onset of symptoms**
  - ✓ Always RT-PCR combined with IgM
  - ✓ Lack of sensitivity in IgM detection might be compensated by the parallel PCR testing

- **Sample taken ≥7 and ≤ 12 days post onset of symptoms**
  - ✓ Always RT-PCR combined with IgM and IgG testing
  - ✓ Lack of sensitivity in IgM detection might be compensated by identification of IgG in previously naive travelers or population

- **Sample taken ≥ 12 days post onset of symptoms**

  IgM and IgG testing

EVD-LabNet

Modified from:
EVD-LabNet Assessment laboratory preparedness and response to chikungunya virus, updated September 2017.

Laboratory diagnosis of CHIK

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- Sample taken ≥ 12 days post onset of symptoms
  - IgM and IgG testing

Remarks:

- Due to the observed short period of viremia, negative RT-PCR results should not be used to exclude the diagnosis
- IgM preferably by commercial IFA

EVD-LabNet

Modified from:
EVD-LabNet Assessment laboratory preparedness and response to chikungunya virus, updated September 2017.

Diagnosis of arboviral infections: challenges/solutions

	Challenges	Possible solutions
Serology	Cross-reactivity with same family viruses	Seroneutralization
	Few commercial methods	In house methods
	Lack of reference materials	Networking
Virus isolation	High biocontainment required	BSL3
	Recognition of isolate	Sharing reagents and protocols through networking
Molecular methods	Multiple viruses to address	Genus-specific PCR (first line) + sequencing
	Cross-reactivity with same ìfamijily viruses	Use of multiple complementary methods, confirmatory tests (e.g.sequencing)

The mission of a reference laboratory

The INMI's Laboratory of Virology has been involved for several years in various aspects of the research and diagnosis of viral infections transmitted by arthropods, in the broader context of emerging infectious agents infections (also unknown)

The Regional Reference Laboratory provides:

- A 24h diagnostic service able to support clinicians in the diagnosis of infections based on uncommon techniques, often not commercially available, requiring advanced technologies or equipments and specific expertise
  - ✓ Diagnosis improvements
  - ✓ Tests for differential diagnosis
  - ✓ Evaluation, development and validation of new tests

- Training / information on relevant diagnostic aspects
- Consulting / operations h24

INMI Laboratories Regional Reference Center for:

- Arboviruses
- Infections in Transplant Patients
- Haemorrhagic fever (Ntl.)
- Molecular advanced diagnostics
- Severe influenza
- Measles and Rubella
- Haepatitis A and E

- Rare infectious diseases [Hansen disease (leprosy), Lyme disease (borreliosis), Whipple disease]
- Streptococcus pneumoniae invasive infections
- Multi-Drug Resistant Tuberculosis
- Health Care Associated Infections

Laboratory of Microbiology

Chikungunya virus diagnostic tools

	Method	Sample	Commercial/in house	Target region	ref
IgM	IFA	serum	In house slides; Euroimmun Chikungunya virus and/or Arbovirus 2		
IgG	IFA	serum	In house slides; Euroimmun Chikungunya virus and/or Arbovirus 2		
Neutralizing antibodies	Microneutralization (limiting dilution)	serum			
Nucleic acids	Real-Time RT-PCR	serum /plasma	in house	nsP3	multiplex with MAYV and ONNV
			in house	E1	Edwards CJ et al. J Clin Virol 2007
			RealStar® Chikungunya RT-PCR Kit 2.0		
Viral isolation	Vero E6, C6-36, BHK-21and RK13 cells	serum			
Molecular characterization (sequencing)	RT-PCR	serum /plasma	in house	nsP1	Hasebe F et al. J Med Virol. 2002
			in house	E1	

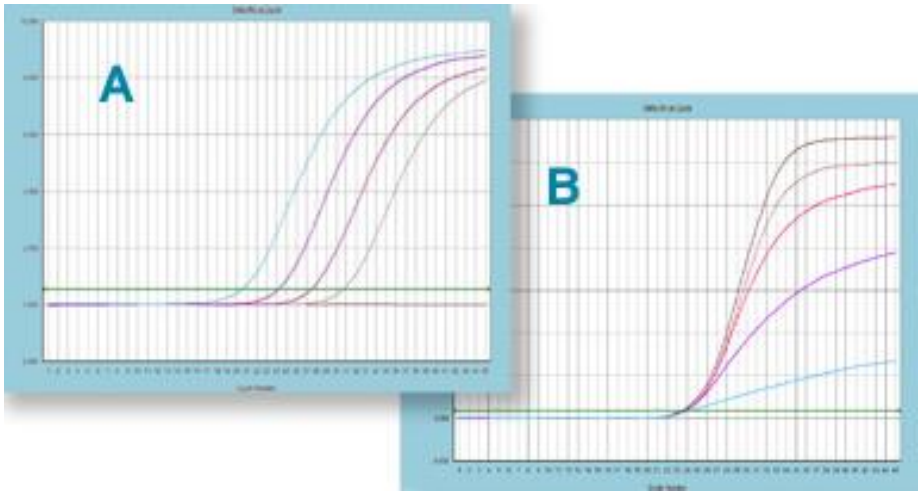
Keep/monitoring competency

- External Quality Assurance

Test	EQA Organizer	2010	2011	2012	2013	2014	2015	2016
West Nile (RNA)	QCMD	X	X	X		X		
	ISS-SISTRA			X	X	X	X	
	ISS-CRIVIB							X
West Nile (sierologia)	ENIVD-CLRN	X	X					
Zika (RNA)	ISS-CRIVIB-MIPI							X
	QCMD							X
	EVDLabNet							X
Chikungunya (RNA)	QCMD							X
Chikungunya (serology)	ENIVD-CLRN					X		
Tick Borne Encephalitis (serology)	ENIVD-CLRN	X	X					
Yellow Fever (RNA)	ENIVD-CLRN	X	X					
Dengue 1-4 (RNA)	ENIVD-CLRN	X		X				
	QCMD				X	X		
Dengue 1-4 (serology)	ENIVD-CLRN				X	X		
Arbovirus (DENV 1-4,CHIKV, ZIKV)	RCPAQAP-WHO							X

Molecular diagnosis algorithm

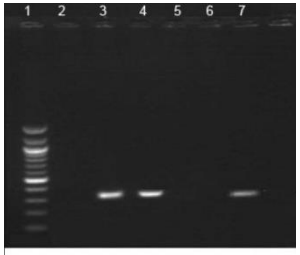
Positive real time RT-PCR



RT-PCR with different target (end point)

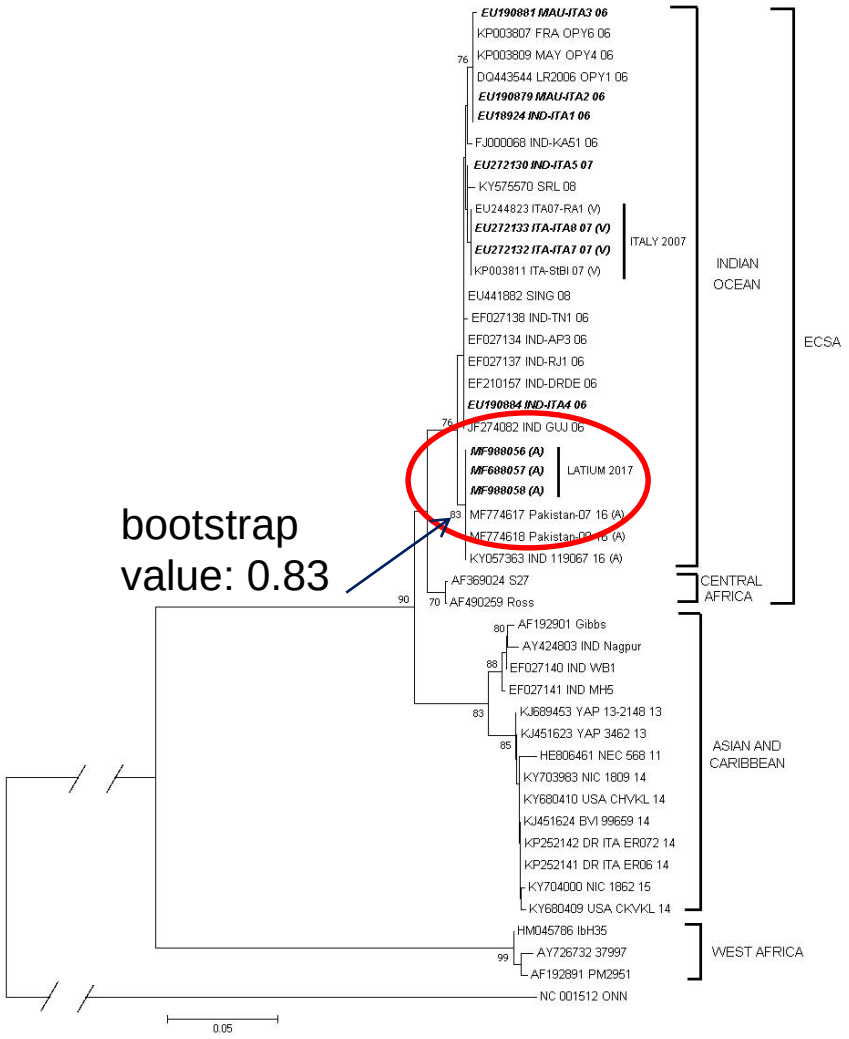
Amplicon sequencing for

- ✓ confirmation and
- ✓ molecular characterization



Lane 1: Molecular marker
Lane 2, 5, 6: Negative for 305 bp
Lane 3 & 4: Patient samples positive for 305 bp
Lane 7: Positive control

Virus characterization 1.



✧ E1 Molecular characterization

The 1st INMI case-patient, was a resident of Anzio with no recent travel history abroad. Admitted to INMI on August 30 with **suspected measles**. Arboviral disease suspected on September 3;
CHIK IgM positive on September 5.

Partial sequence of the E1 coding region directly from clinical samples of 3 patients involved in the current outbreak.

GenBank accession numbers: MF988056–MF988058.

Bordi L, et al. Emerg Infect Dis. 2018

Arboviral infection diagnosis: integrated approach

- For the multiplicity of pathogens causing similar symptoms (syndromic approach), and the different kinetics of diagnostic markers, a diagnostic panel integrating multiple parameters is necessary (serology, PCR, sequencing, virus culture….)

Differential diagnosis

Viruses

- ✓ **Flavivirus:** Yellow Fever, Zika, West Nile Fever, Dengue 1-4
- ✓ **Alphavirus:** Mayaro, O'nyong-'nyong, Sindbis
- ✓ **Other: Measles**, enteroviruses, Rubella, **Parvovirus B19**, Flu, Haemorrhagic fever (Arenaviridae, Filoviridae, Bunyaviridae) Adenovirus, Ross River fever, Hantavirus.

Parasitic infections

- ✓ Malaria

Bacterial infections

- ✓ Rickettsiosis
- ✓ Sistemic N.gonorrhoeae
- ✓ Reactive post infective artheritis
- ✓ Group A Streptococcus
- ✓ Leptospirosis

Virus characterization 2.

✧ Virus isolation and further molecular characterization

Several isolates were obtained on BHK-21 and RK-13 cells from patients with different clinical presentation

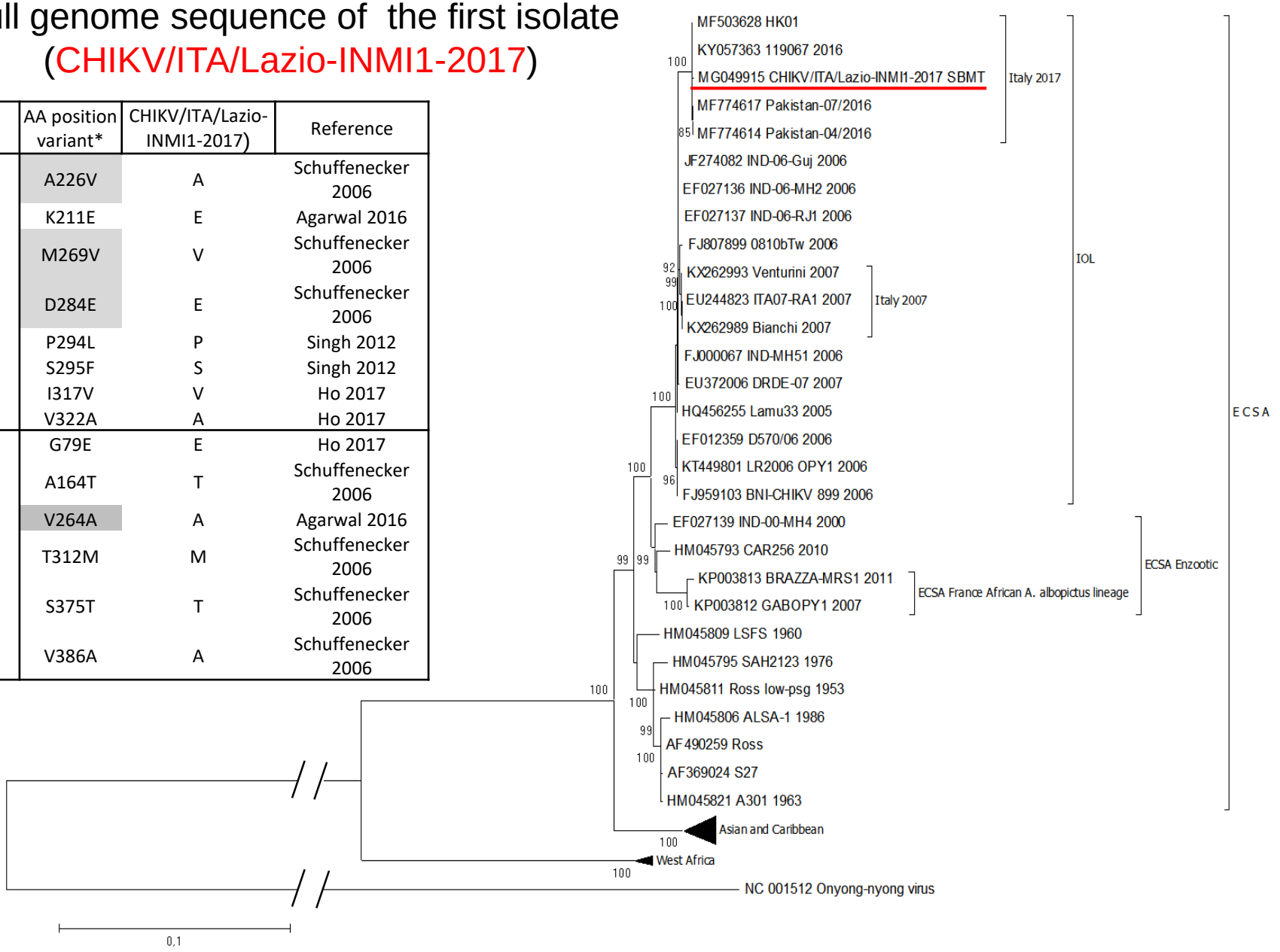
First isolate *CHIKV/ITA/Lazio-INMI1-2017* was adapted on Vero E6 cells; complete genome sequence submitted to GenBank (accession number: MG049915)

Viral preparations will be available on the EVAg website for sharing with other laboratories, for the establishment of EQA and for assay validation (e.g. for blood screening purpose)

Virus characterization 2.

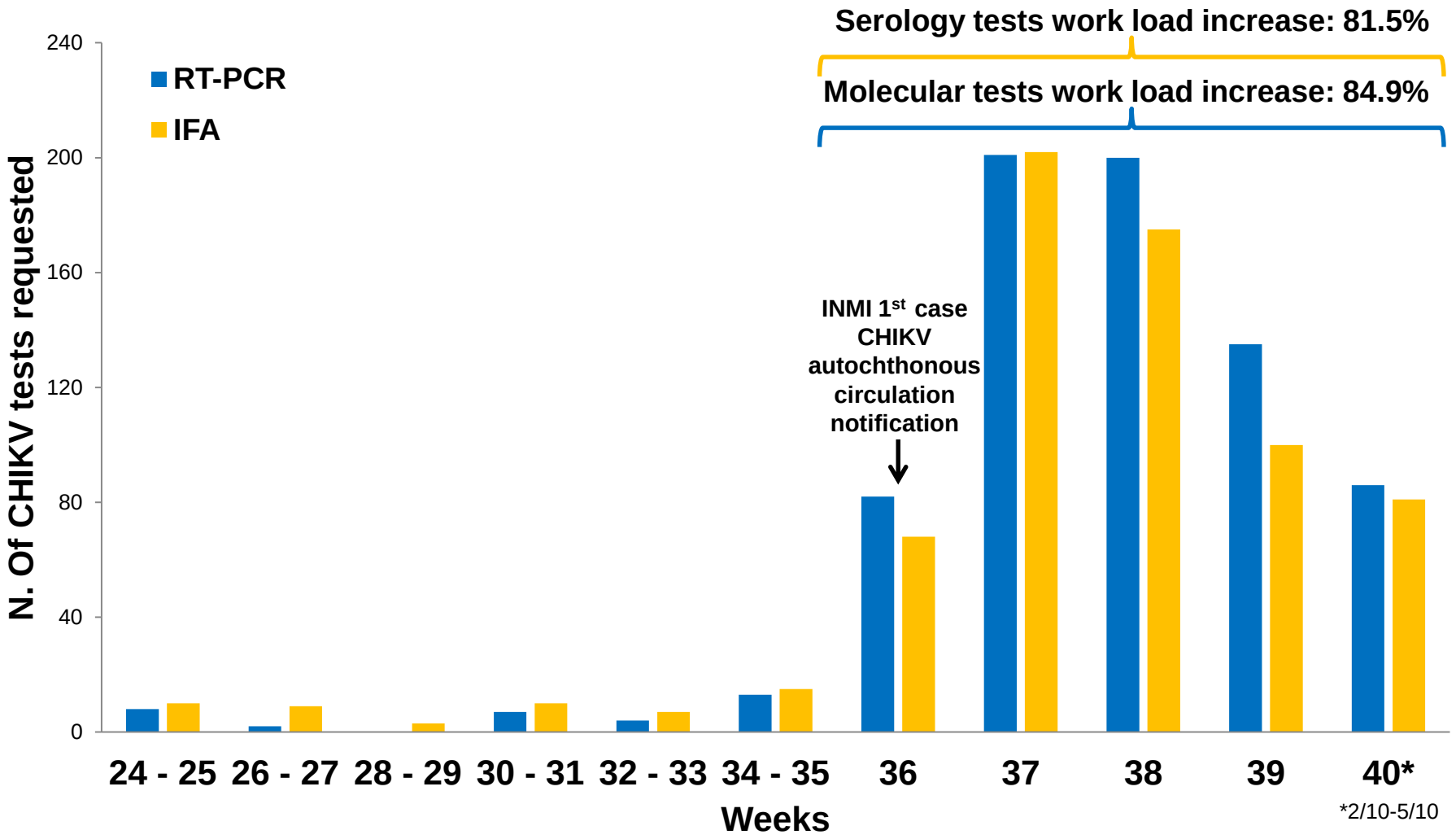
Full genome sequence of the first isolate (CHIKV/ITA/Lazio-INMI1-2017)

	AA position variant*	CHIKV/ITA/Lazio-INMI1-2017)	Reference
E1	A226V	A	Schuffenecker 2006
	K211E	E	Agarwal 2016
	M269V	V	Schuffenecker 2006
	D284E	E	Schuffenecker 2006
	P294L	P	Singh 2012
	S295F	S	Singh 2012
	I317V	V	Ho 2017
	V322A	A	Ho 2017
E2	G79E	E	Ho 2017
	A164T	T	Schuffenecker 2006
	V264A	A	Agarwal 2016
	T312M	M	Schuffenecker 2006
	S375T	T	Schuffenecker 2006
	V386A	A	Schuffenecker 2006



Carletti F et al. Genome Announc in press

Laboratory Workload Impact



During the current surveillance period (June 15 to October 13) we analyzed 858 samples (676 sera/plasma, 182 samples from different body districts) for the presence of CHIKV-RNA and 756 sera for the presence of CHIKV specific IgG and IgM.

Laboratory Workload Impact

Regional health authorities reiterated the appointment of INMI virology laboratory as the regional reference center to better coordinate outbreak management

- Sample load (mean daily routine samples 190 + CHIKV samples 24 [rangie 2-69])
- Impact on laboratory routine (samples sending instructions improving, samples check-in, processing, RNA extraction, testing, reporting, aliquoting and…answer to information requests….)
- Test supply
- Rapid communication issues

We doubled personnel dedicated to sample check-in and RNA extraction, to be able to give results in 48 hours, to produce CHIKV slides (3 batches of60 units) within 10 days, to speed communication with health authorities and local units

Laboratory report times and information flow

- Laboratory report time (mean): 48 hours
- Information flow:
  - ✓ Hospitals (emergency room or inward Infectious diseases units)
  - ✓ In case of requests from outpatients'clinics, the report is sent directly to Sub-provincial health authorities
- Daily cross-check with the SERESMI (Regional Infectious Diseases Surveillance and Response System)
- Samples aliquoted and stored at the INMI Biorepository for retrospective evaluation and to be sent to the National Reference Laboratory

Some laboratory data

- CHIKV RT-PCR results vs symptoms onset
  - **93%** RT-PCR positive ≤7 days
  - **7%** RT-PCR positive >7 days (up to 40 days)

- CHIKV RT-PCR* positive - average threshold cycle (Ct)

Symptoms onset (days)	Ct value mean	SD	Range
0 – 7 total	30,73	3,07	9-42
0 -7 IgM **-**	19,61	5,61	9-32
0 -7 IgM **+**	31,95	5,64	15-42
8 - 40	35,83	8,52	30-43

* RealStar® Chikungunya RT-PCR Kit 2.0

Monitoring T-cell immunity

- Total lymphocyte counts
- $CD4^+$ and $CD8^+$ T cell counts
- CD4 response to PHA (Cylex)

Non specific
- phenotypic and
- functional parameters

- Tetramer Ag-staining
- Intracellular IFN-γ (flow cytometry, Ag-specific cellular subsets)
- Quantiferon (antigen-specific $CD8^+$ T-cell response)
- Elispot (antigen-specific $CD4^+$ and $CD8^+$ T-cell response)
- Cultured-ELISPOT (central memory antigen-specific $CD4^+$ and $CD8^+$ T-cell response)

Virus-specific
- phenotypic and
- functional parameters

Role of RRL in the surveillance of arboviral infections in solid-organ transplantation and blood donation

In the context of the current outbreak, from September 9 screening for CHIKV infection was required for solid organ donors, included corneal tissue, and HSC donors

As of Nov. 8, samples from 122 subjects were evaluated and all were negative for CHIKV RNA.

- Among them one resulted positive for IgG (1:320) and IgM (1:40)
- The donor was from Venezuela were Chikungunya is still circulating
- She tested also IgG positive and IgM negative for flaviriruses

Blood Units
Study on feasibility and validation of a NAT test for CHIKV

REGIONE LAZIO

Direzione Regionale Salute e Politiche Sociali

OGGETTO: valutazione di applicabilità test diagnostico per ricerca molecolare del virus Chikungunya

In relazione alla vostra nota di pari oggetto, prot. n.2106 del 14.9 u.s., si comunica che, di concerto con il Centro Regionale Sangue, il centro di riferimento individuato per la validazione del test è il laboratorio dell'Ospedale Sandro Pertini che già svolge la funzione di centro regionale per la qualificazione biologica degli emocomponenti e che si potrà avvalere del supporto scientifico dell'Istituto Nazionale malattie Infettive Lazzaro Spallanzani

Cordialmente

IL DIRETTORE
(Dr. Vincenzo Panella)

To overcome the issue of stopping blood donation in affected territories, National Blood Center (CNS) considered molecular screening of blood units

- ✓ It was established to assess feasibility and sustainability, and to perform validation of a molecular test for CHIKV
- ✓ Appointed: INMI virology lab, regional laboratory for NAT on blood units, ISS-CNS lab

SISTEMA SANITARIO REGIONALE
CENTRO REGIONALE SANGUE

REGIONE LAZIO

Arboviral infection diagnosis: integrated approach

- For the multiplicity of involved viruses and the different kinetics of diagnostic markers, a diagnostic panel integrating multiple parameters is necessary (serology, PCR, sequencing, virus culture….)

- To overcome the general scarcity of standardized (commercial) methods, the laboratory must be integrated in networking circuits, also useful to expand the panel of available diagnostic/confirmatory tests (virtual global laboratory)

Concetta Castilletti
Fabrizio Carletti
Licia Bordi
Eleonora Lalle
Francesca Colavita
Claudia Caglioti
Donatella Vincenti
Chiara Agrati
Antonino Di Caro
Biorepository team

Laboratory people

Emanuele Nicastri
& other physicians

Clinical Department

Paola Scognamiglio
Francesco Vairo

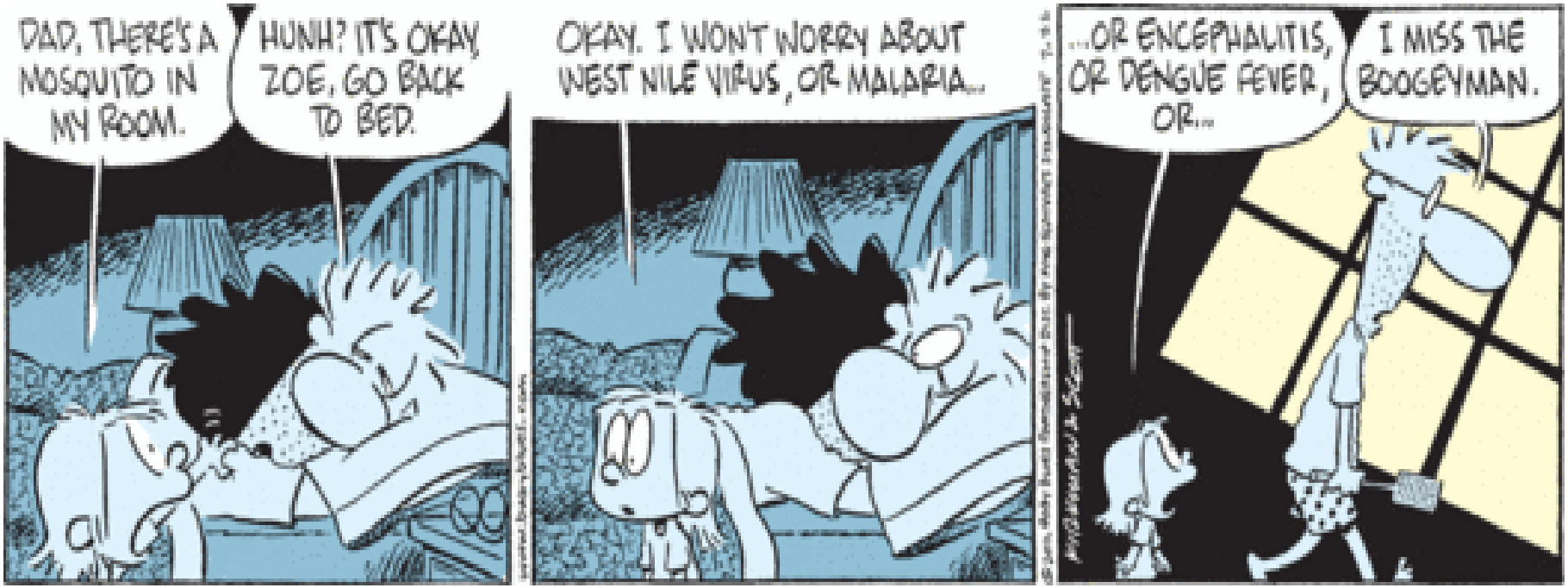
SERESMI

Giuseppe Ippolito

Scientific Direction

National Institute
for Infectious Diseases
"L. Spallanzani"
Rome, Italy

Thank you


DAD, THERE'S A MOSQUITO IN MY ROOM.
HUNH? IT'S OKAY, ZOE, GO BACK TO BED.
OKAY. I WON'T WORRY ABOUT WEST NILE VIRUS, OR MALARIA...
...OR ENCEPHALITIS, OR DENGUE FEVER, OR...
I MISS THE BOOGEYMAN.

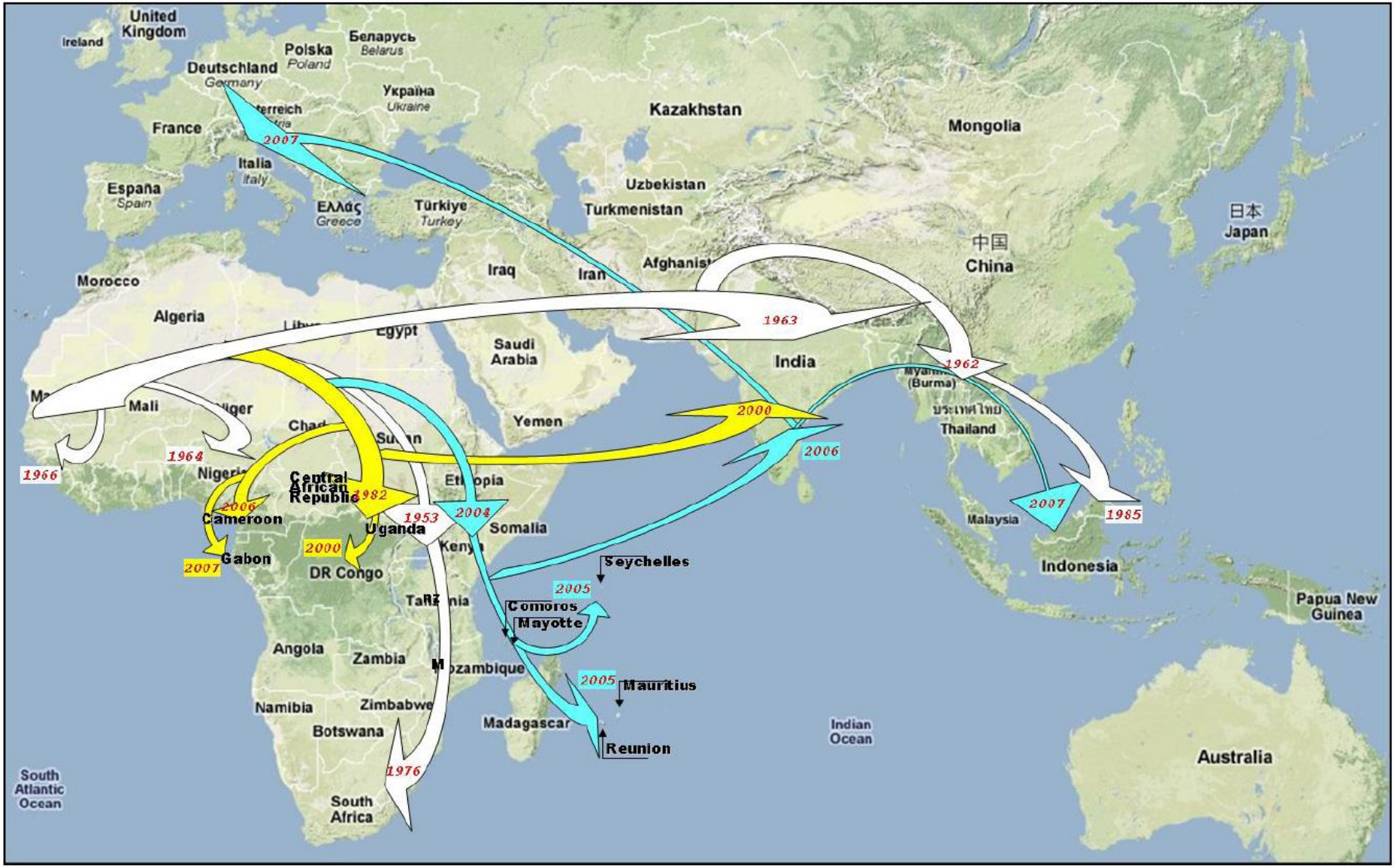
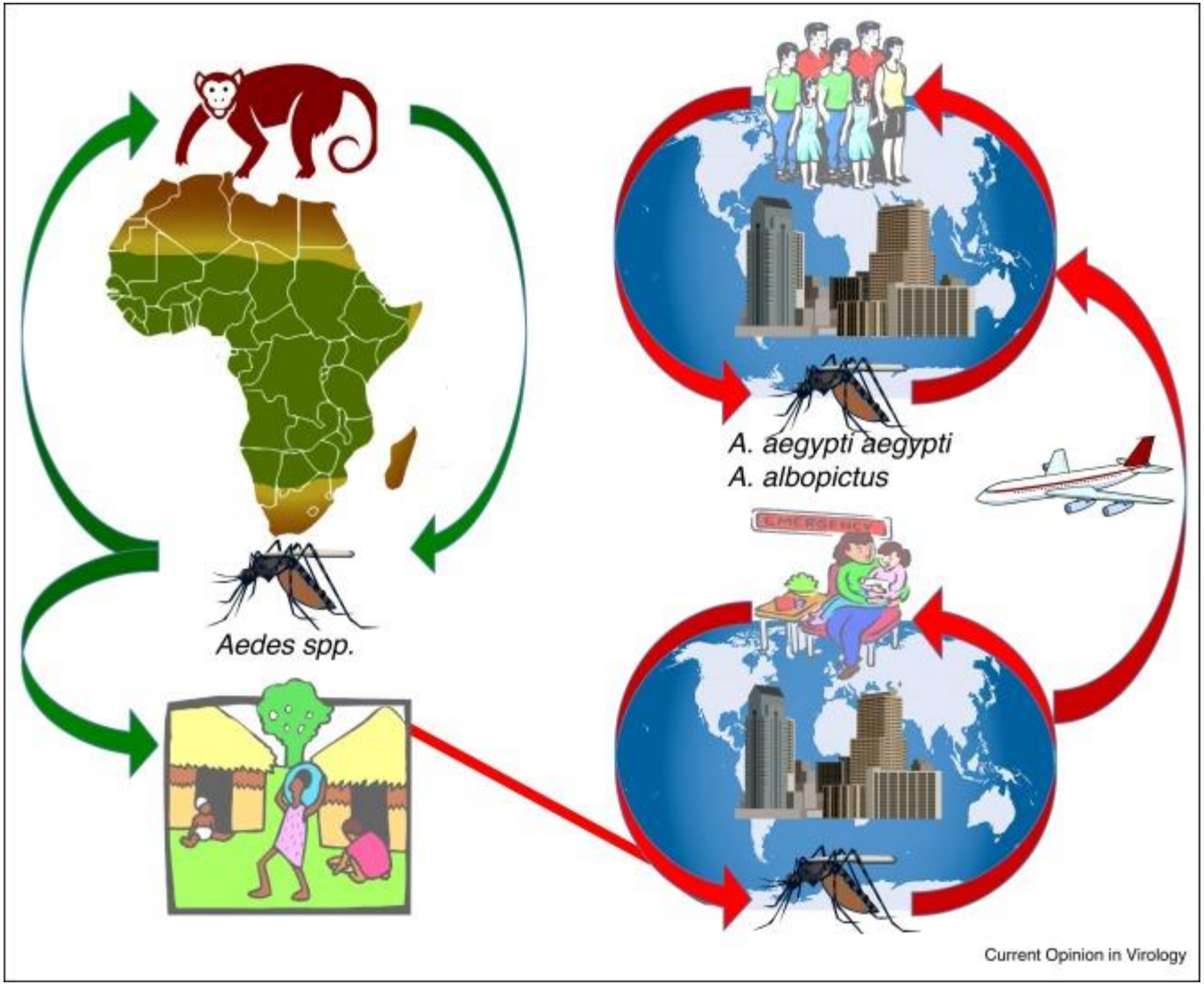
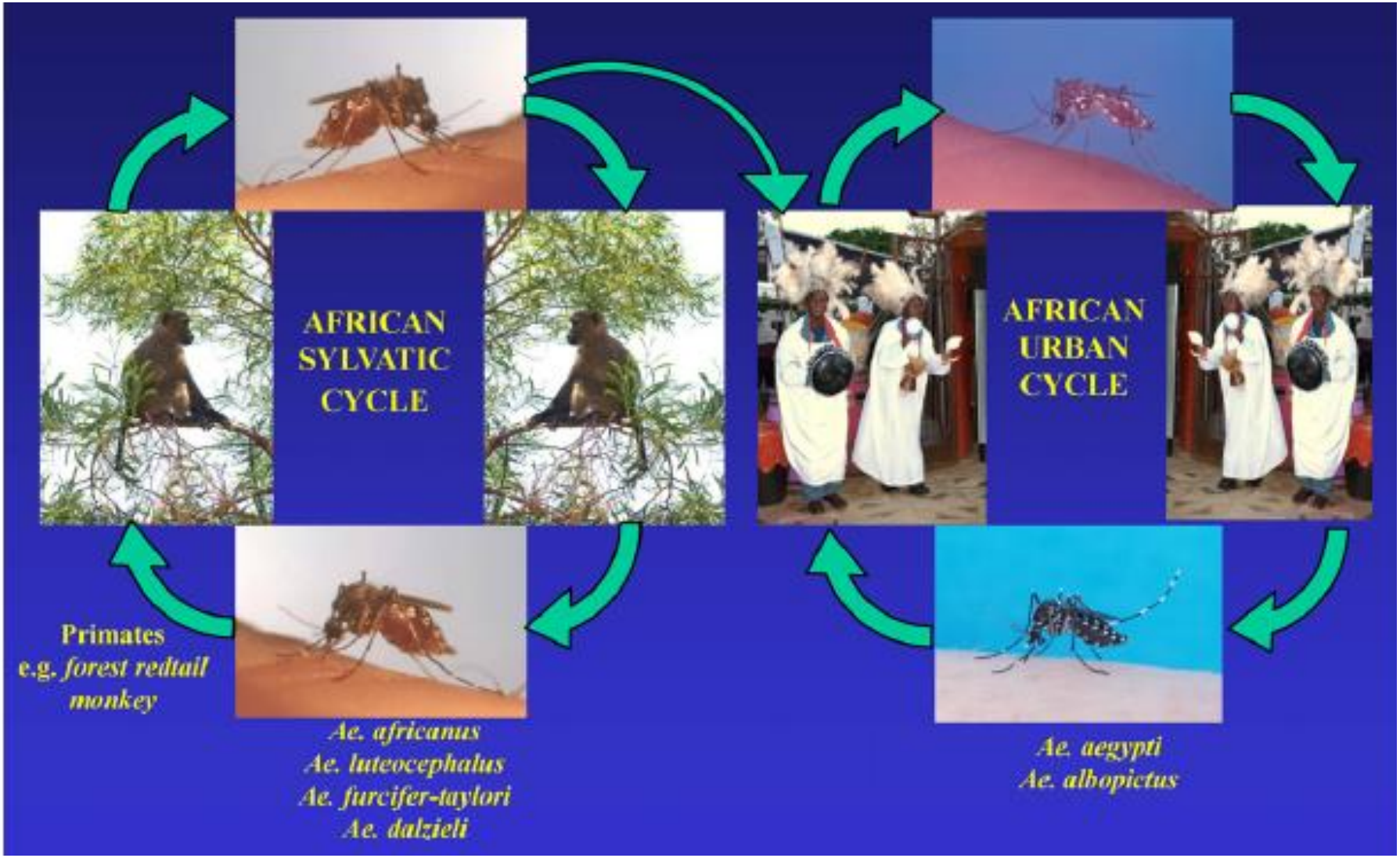


Figure 2
Predicted dispersal pattern of Chikungunya virus from Africa to the Indian Ocean and Europe during the past 20 to 50 years.

de Lamballerie X, Virology J 2008



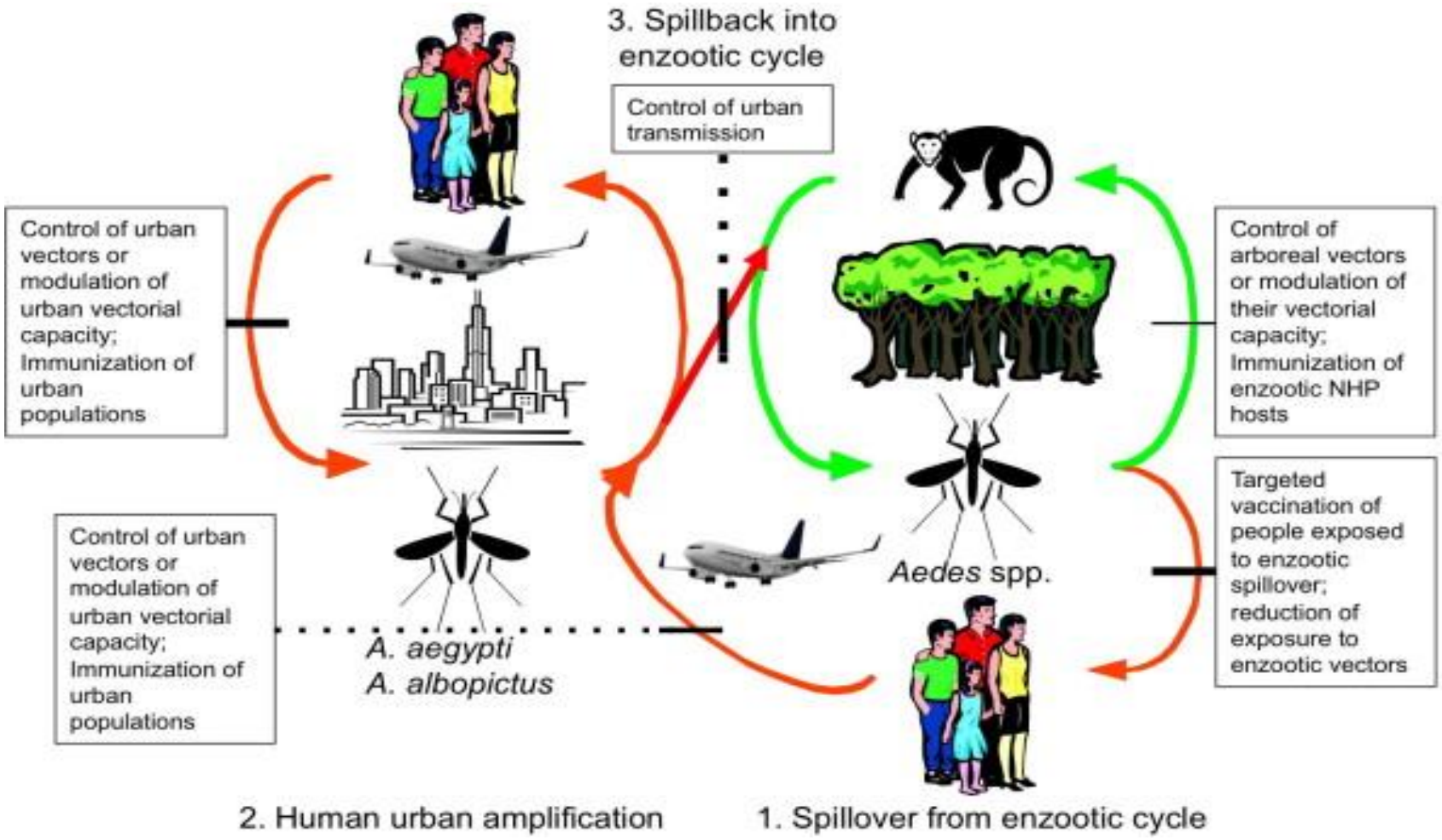
Tsetsarkin KA, Curr Opin Virol 2016



Chikungunya virus exists in Africa in a forest cycle involving baboons and other primates and forest species of mosquitoes. It can also be transmitted in a human-mosquito-human cycle by *Aedes aegypti*.

Gould EA, Higgs S. *Trans R Soc Trop Med Hyg* (2008),

Enzootic and urban CHIKV transmission cycles



Weaver SC, Antiviral Res 2015

CHIK dissemination after mosquito bite

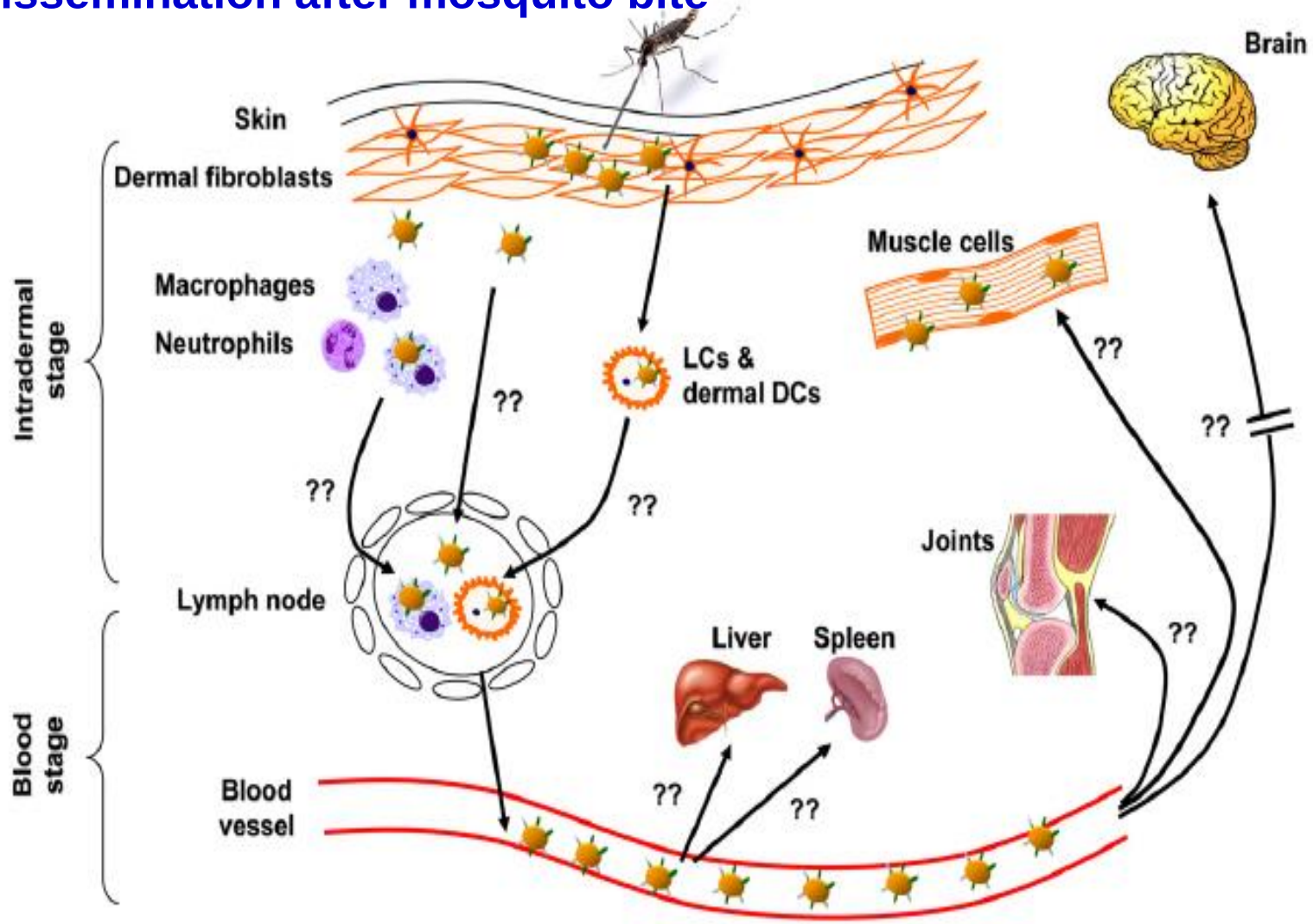
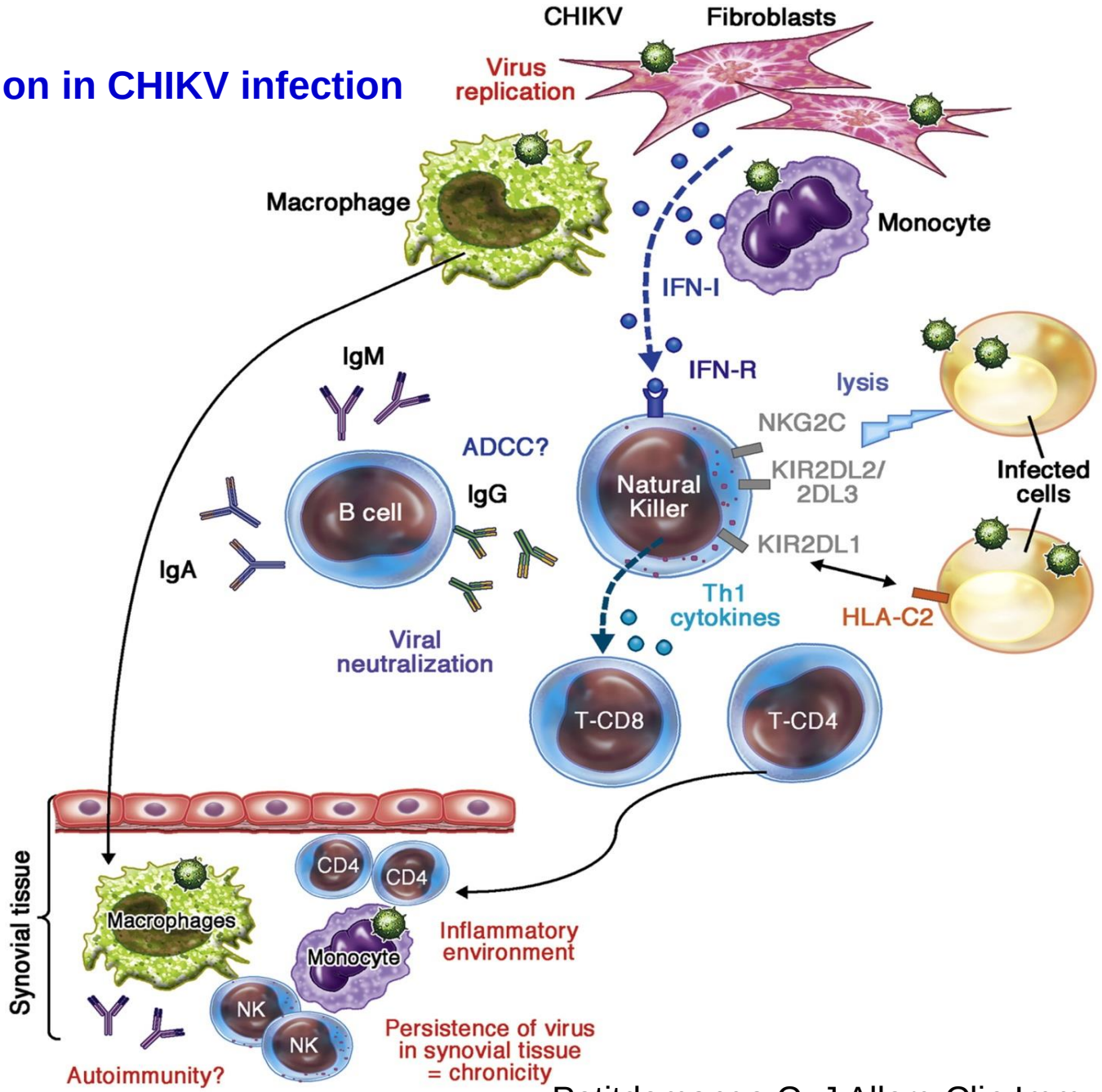


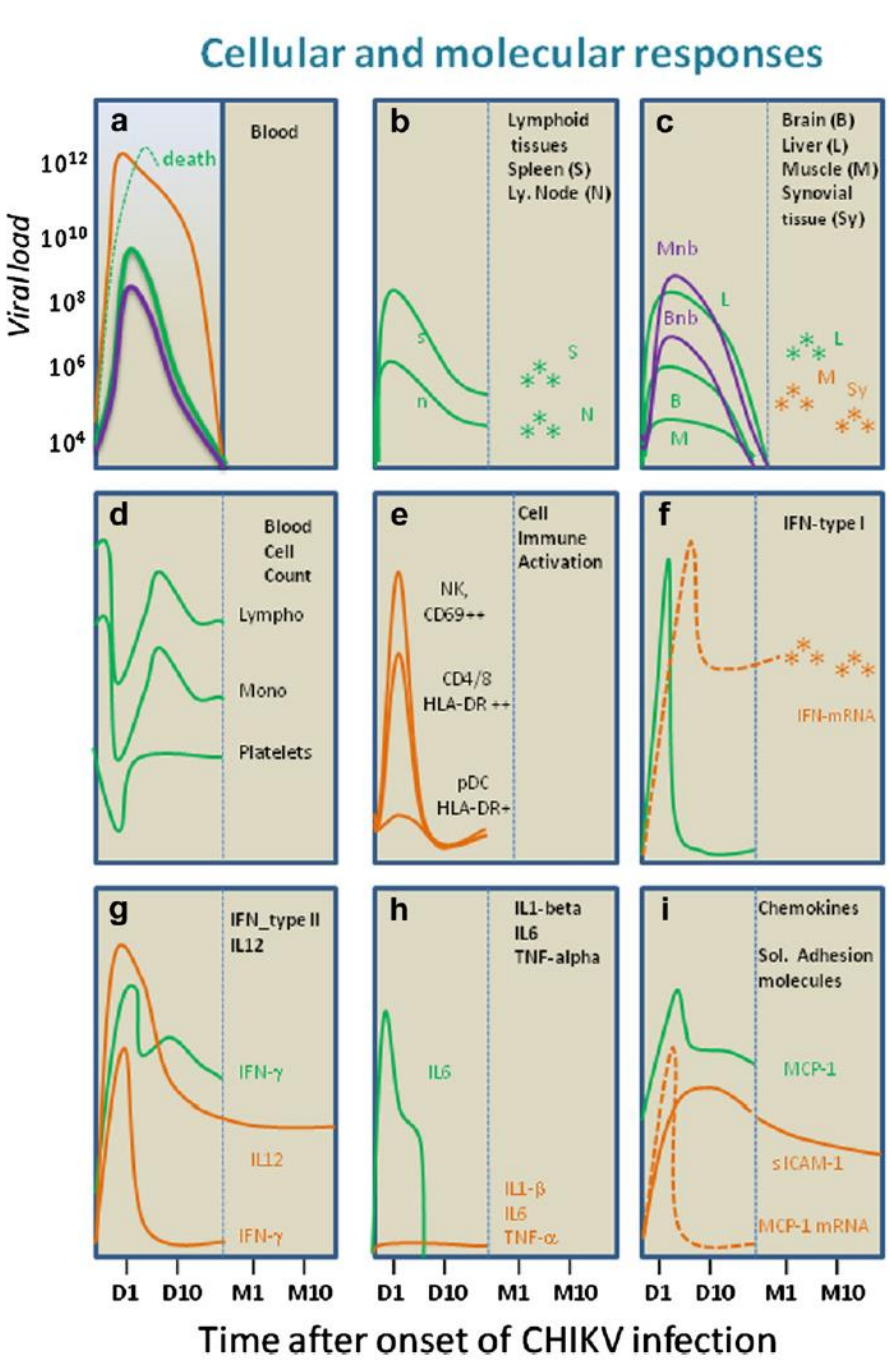
Fig. 1. Schematic representation of CHIKV dissemination after mosquito bite. *Aedes* mosquito inoculates CHIKV into intradermal compartments. Resident immune cells in the intradermal layer include dendritic cells (DCs), Langerhans cells (LC), macrophage (Mφ) and neutrophils that can interact and contain CHIKV from further virus dissemination. CHIKV-infected cells may migrate to the draining lymph nodes and trigger innate immune response for virus elimination. However, virus escaped from lymph nodes may release into blood circulation and disseminate to various parts of the body.

Kam Y-W. et al Microb Infect 2009

Immune activation in CHIKV infection

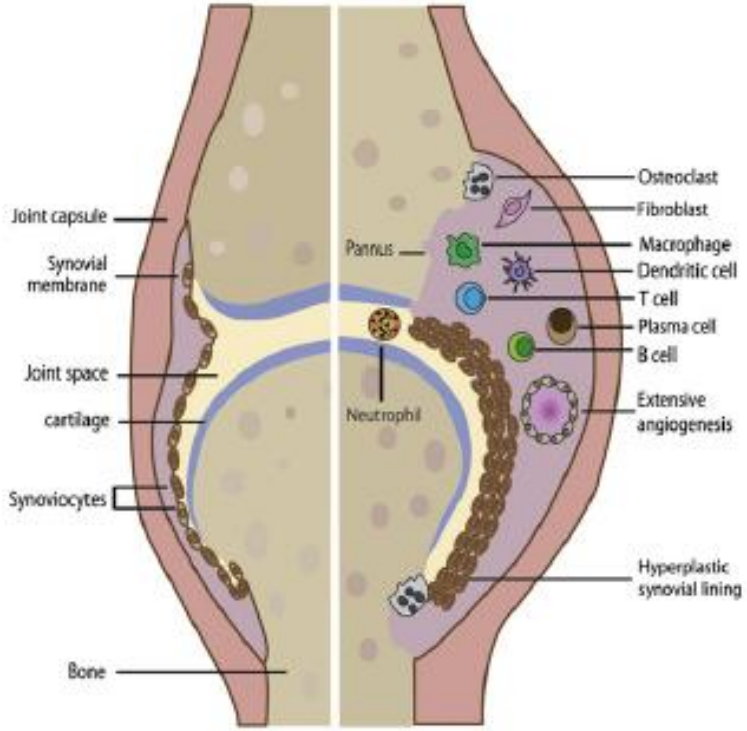


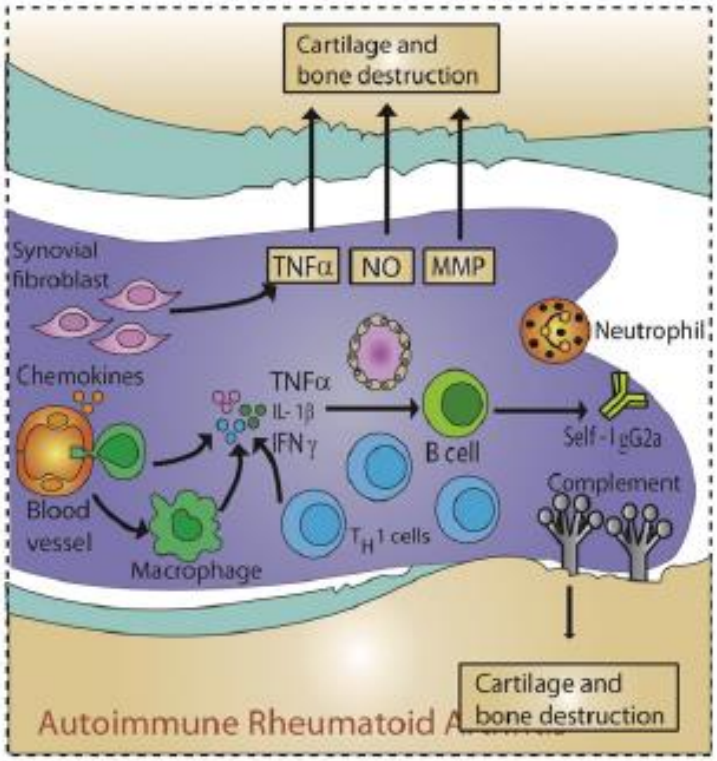
Petitdemange C, J Allerg Clin Immunoll 2015



- a: viral load in blood
- b,c: viremia in tissues and detection of viral antigens and/or RNA as an indication of viral persistence (stars)
- d: blood cell count
- e: immune cell activation
- f: detection of type I IFN in serum and expression of IFN-α mRNA in PBMCs from infected patients with chronic arthralgia (dashed line with stars)
- g: levels of IFN-γ and IL12 in blood
- h: low levels of proinflammatory cytokines (IL1b, IL6, TNF-α) in the acute phase of CHIKV
- i: levels of MCP-1 (CCL2) (mRNA in PBMC as a dashed line), soluble ICAM-1 (surrogate marker of leukocyte trafficking through activated endothelial cells)

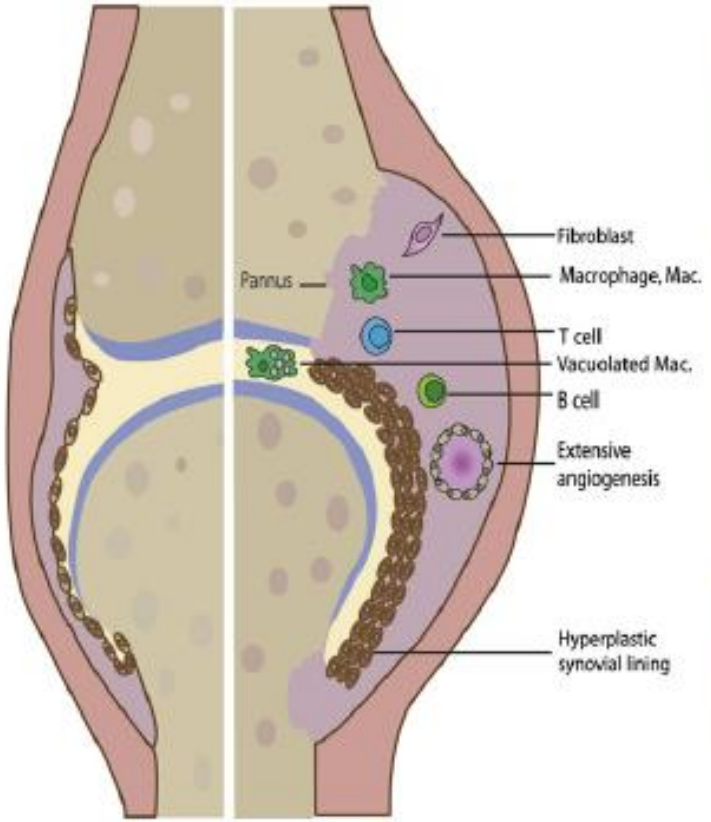
Jaffar-Bandjee ME et al. Microbes Infect 2009

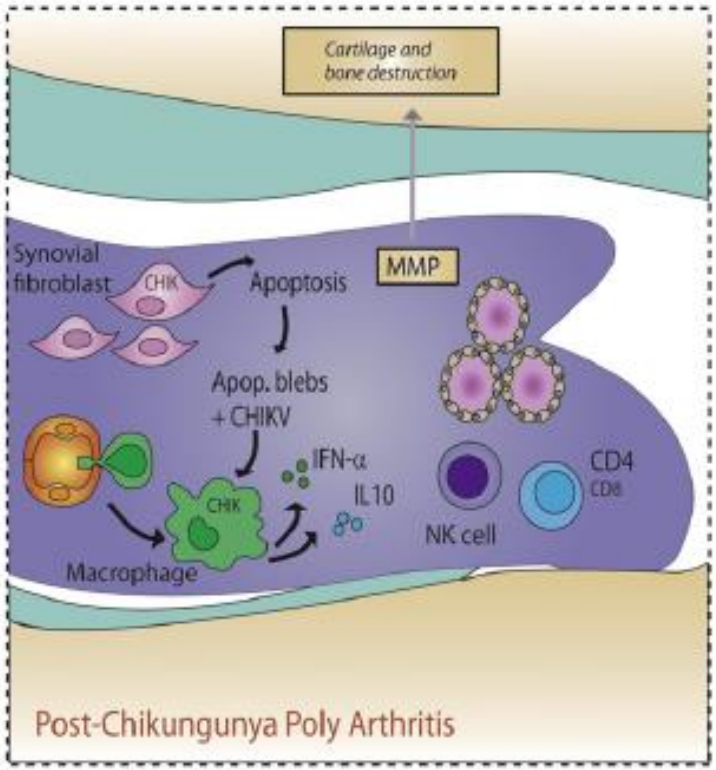




Reumathoid artritis

- Activated neutrophils, CD4 and B plasma cells
- Many pro-inflammatory cytokines,
- Bone destruction driven by TNF-alpha, NO and MMP
- Anti CCP+ anti RF





Post Chik artritis

- Few T cells
- B cells and neutrophils not involved
- Inflammatory response >>IFN alpha + IL10
- No other pro-inflammatory cytokines
- No Anti CCP+ anti RF

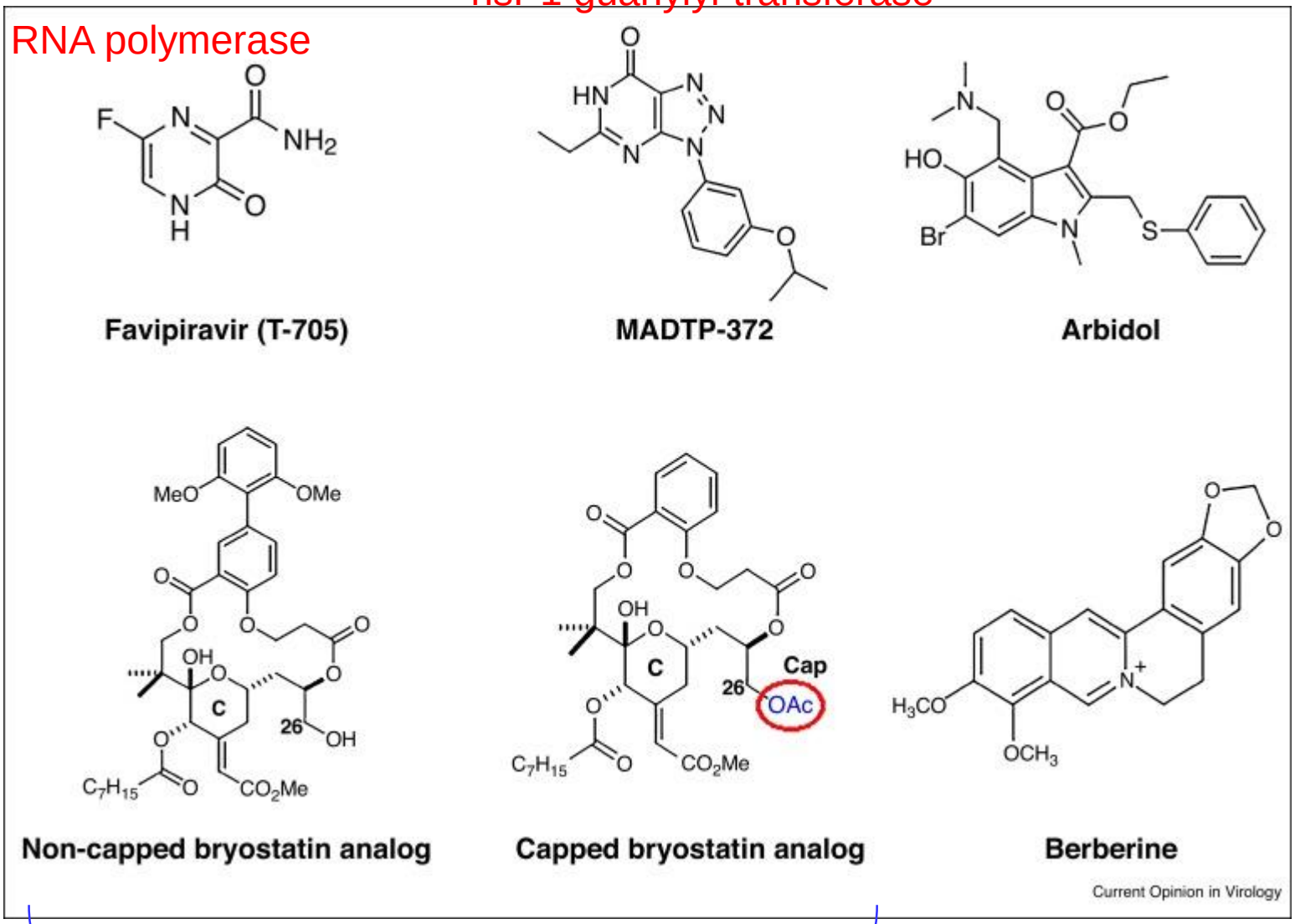
Chemical structures of selected inhibitors of Chik virus replication

Viral targets

RNA polymerase

nsP1 guanylyl-transferase

E2 (fusion)



Host targets

PKC

MAPK signaling pathways

Abdelnabi R et al. Curr Opin Virol 2017

Acquisition of cholesterol dependence coincides with adaptation to A. albopictus

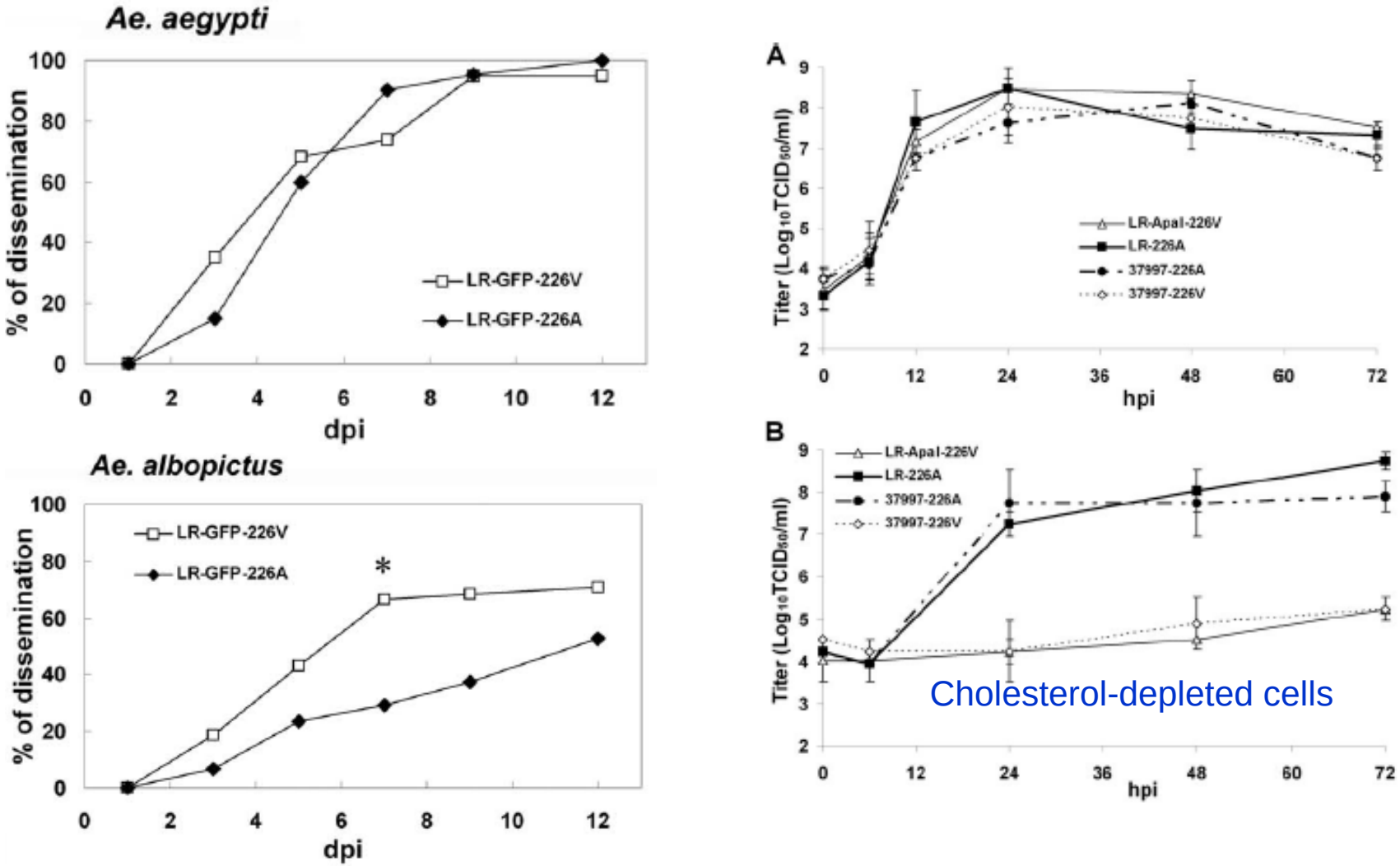
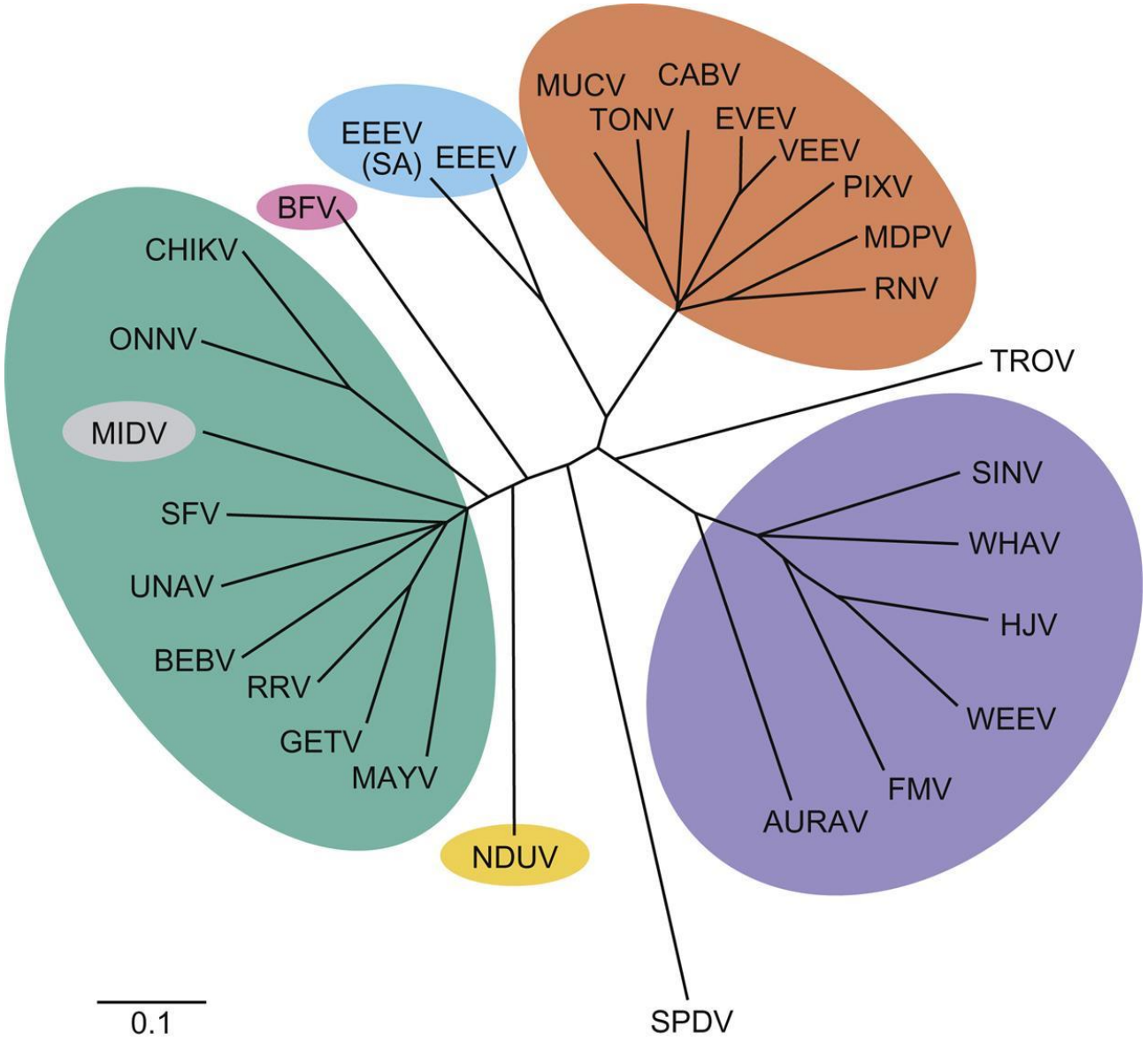


Figure 6. Effect of E1-A226V Mutation on *In Vitro* Growth of CHIKV in Standard (A) and Cholesterol-Depleted (B) C6/36 Cells

Tsetsarkin KA et al. Plos Pathogens 2007

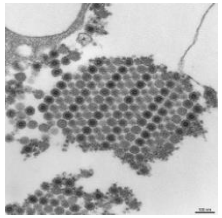
Unrooted phylogenetic tree of representative isolates of all alphavirus species generated from the E1 nucleotide sequences Antigenic complexes are indicated by color circles.

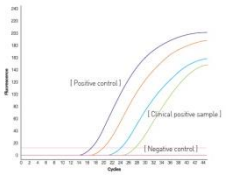


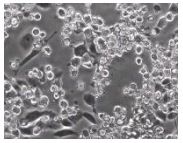
https://veteriankey.com/togaviridae-2/

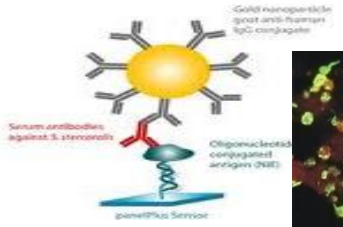
INMI - L. Spallanzani

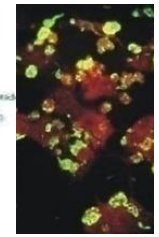
Summary of diagnostics: Clinical samples overall strategy

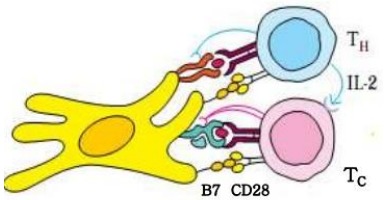












- **Direct detection:** light microscopy, electron microscopy, etc
- **Pathogen isolation:** viral isolation on cell cultures for strain characterization and/or further studies
- **Molecular detection:** for rapid diagnostic orientation, **group specific PCR or RT-PCR** followed by pathogen type identification by type-specific RT-PCR and/or sequencing

Direct methods

- **Immunological methods:** detection of pathogen-specific antibodies; not suitable for rapid diagnosis; important for confirmation of diagnosis and for surveillance (when available)
- **Cell mediated immunity**: Th or Tc response to pathogen or with peptides, for evaluation of pathogenetic aspects

Indirect methods

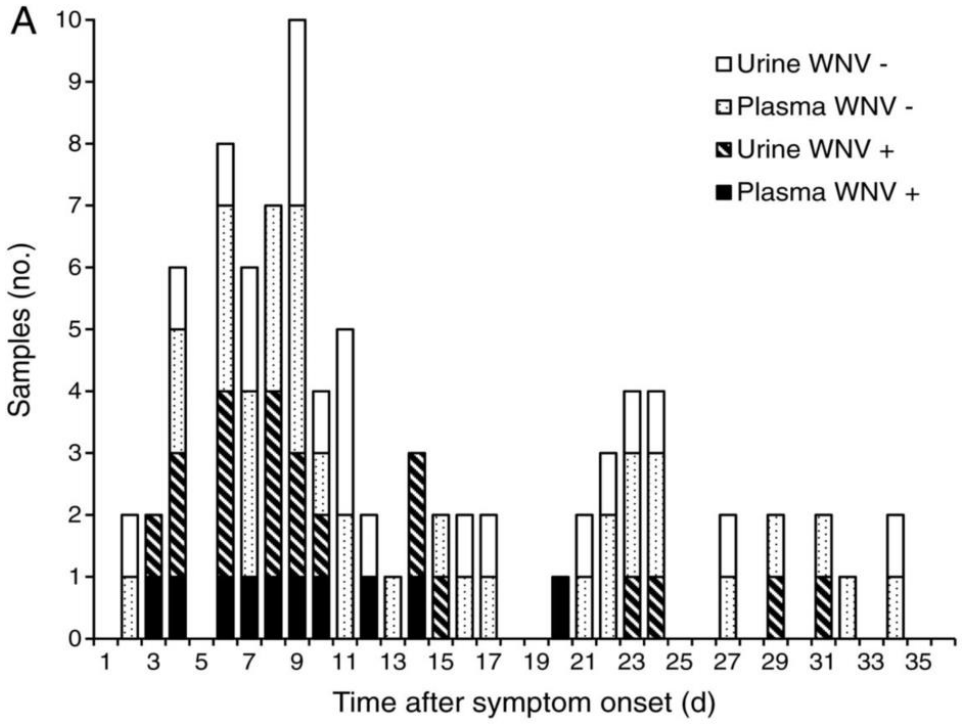
Laboratory diagnosis of CHIK

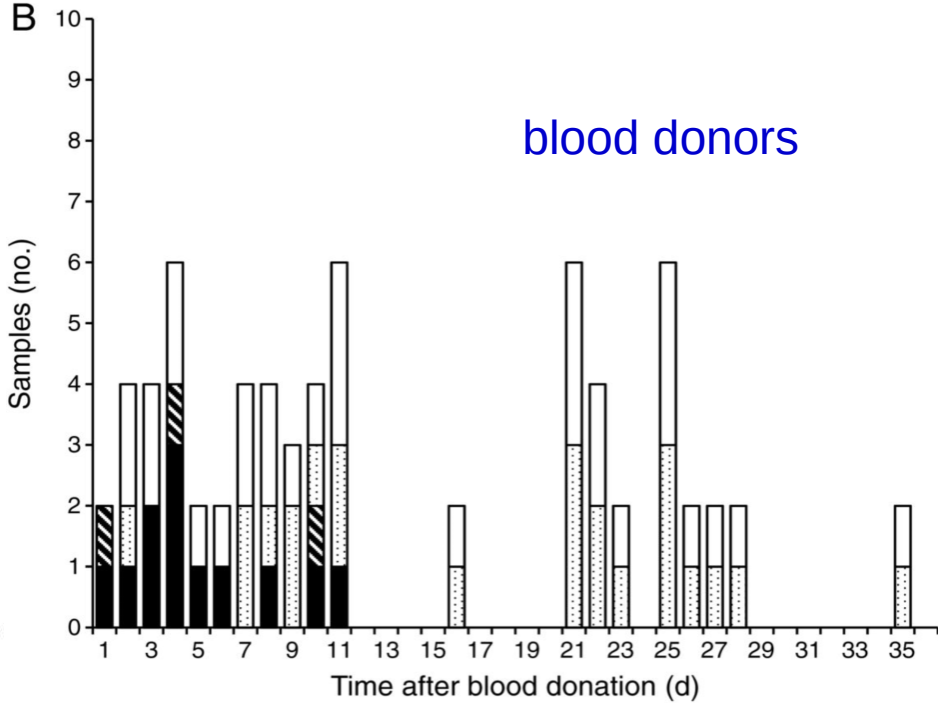
Selection of optimal sample type(s)

The experience with other arboviruses

In patients with West Nile neuroinvasive disease or West Nile fever, WNV RNA was detectable in urine more frequently and for a longer time than in plasma, while in blood donors the detection rate of WNV RNA in urine was lower than in plasma

patients with West Nile disease





blood donors

Barzon L et al. J Infect Dis. 2013

??????

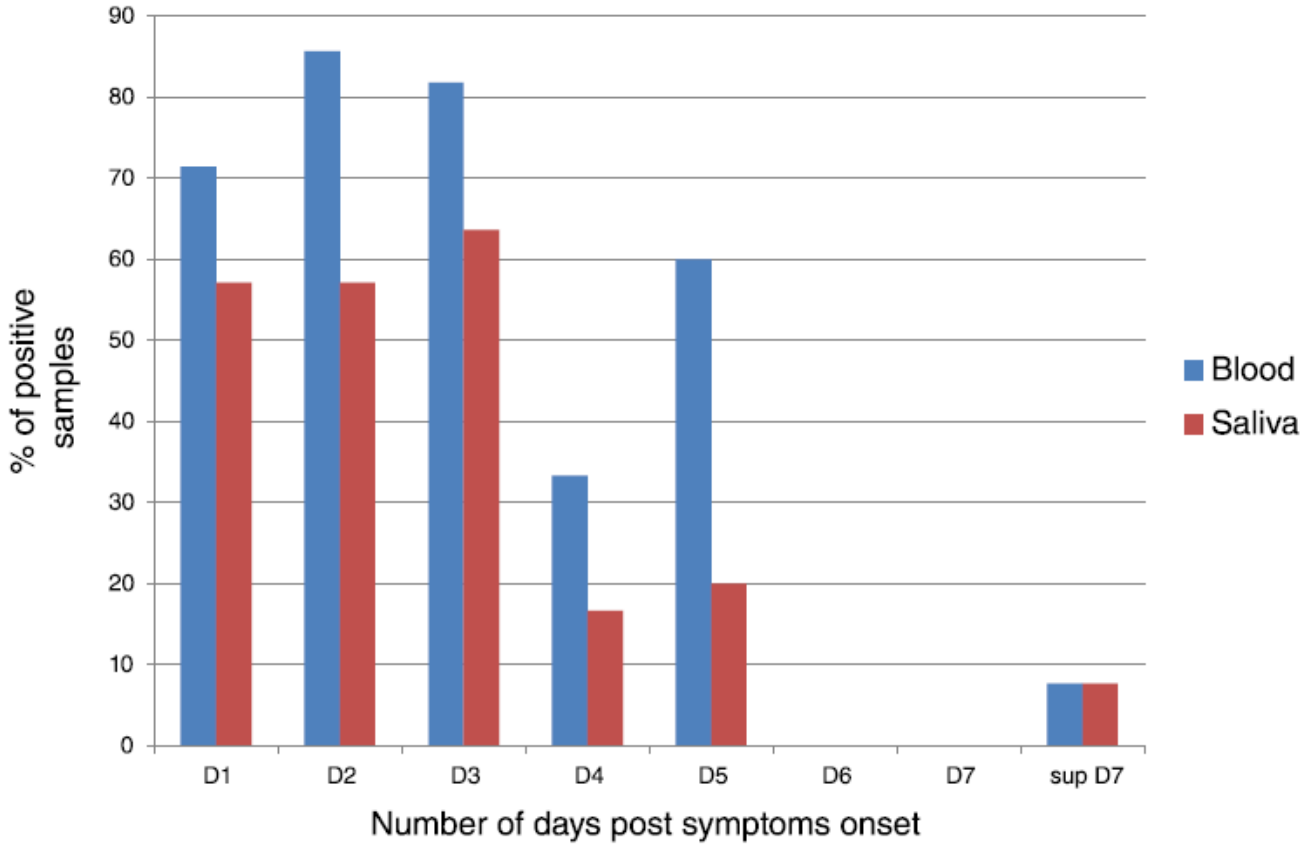
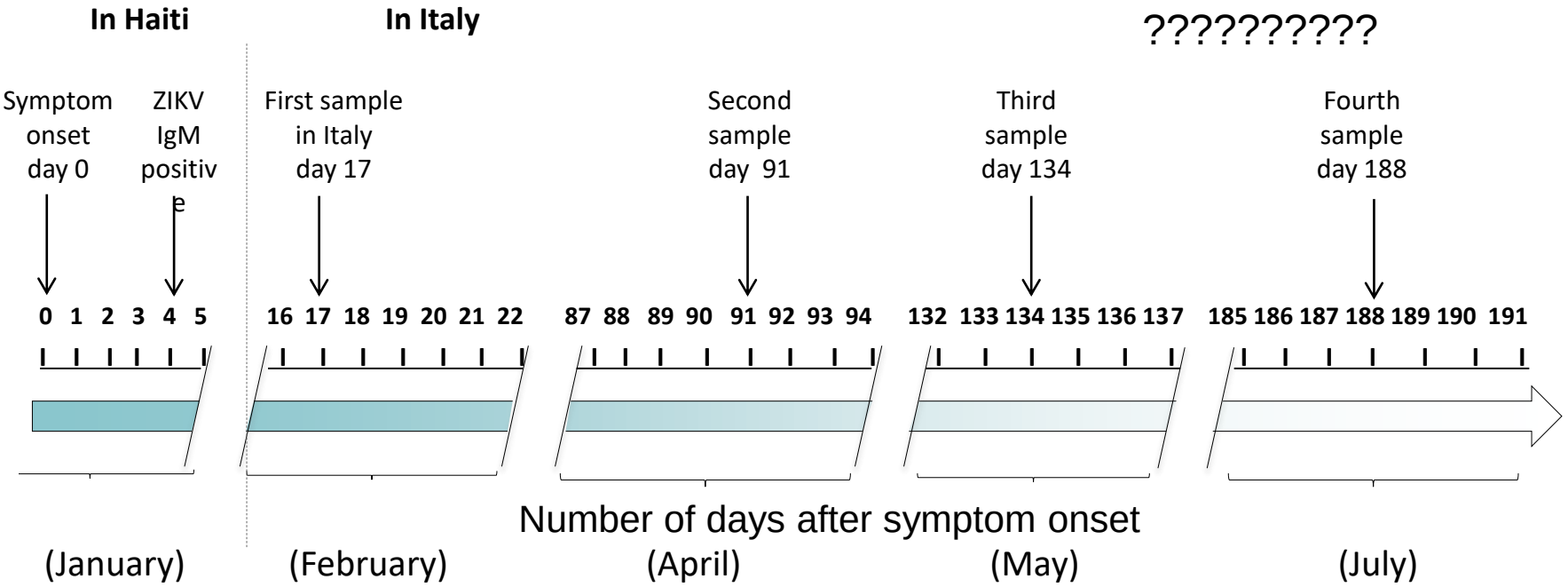


Fig. 1 Proportion of positive samples according to the number of days after symptoms onset for the 60 patients with both blood (blue) and saliva (red) samples tested by ZIKV RT-PCR

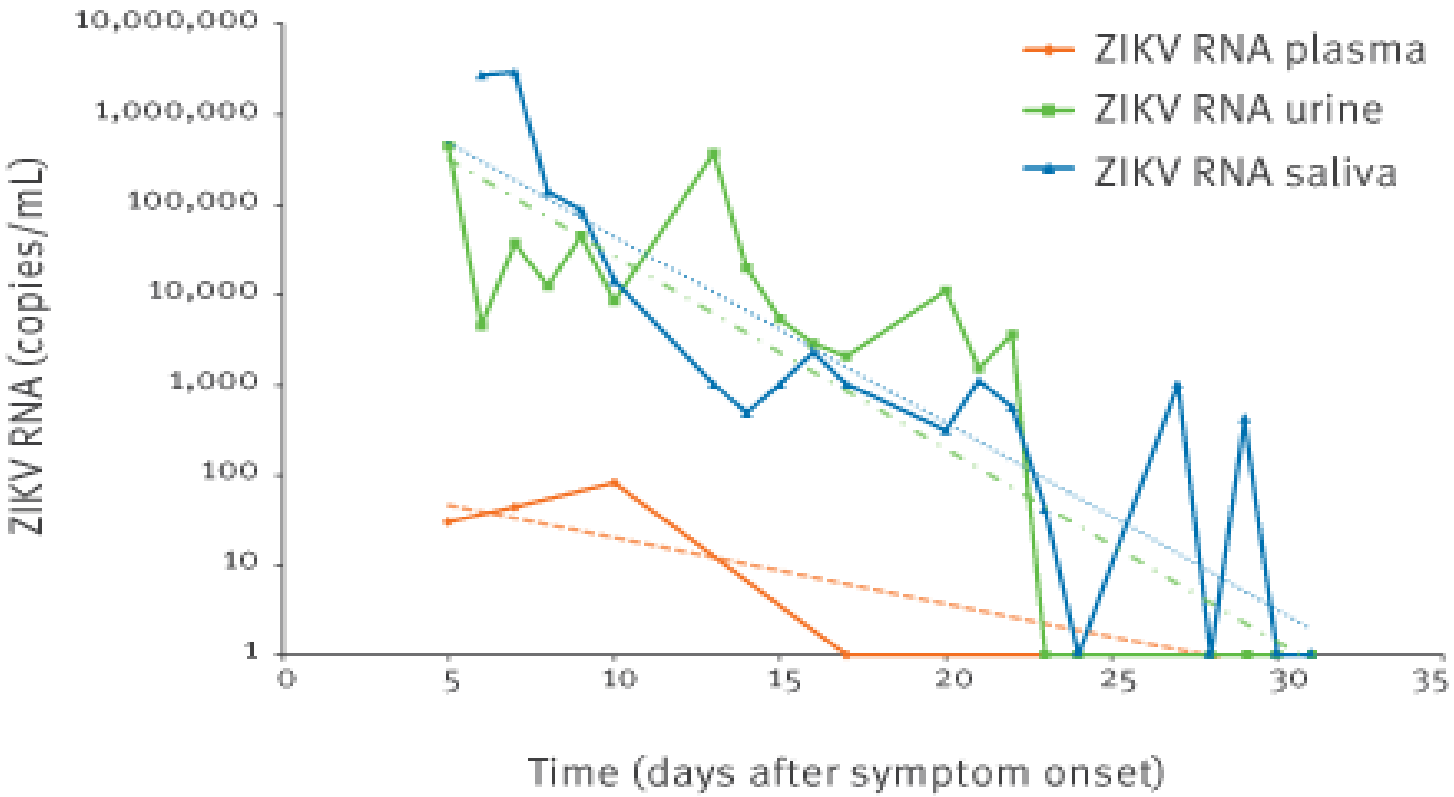
Musso D et al. Virol J 2016



Type of test and sample	Results			
	Day 17[a]	**Day 91[a]**	**Day 134[a]**	**Day 188[a]**
ZIKV real-time RT-PCR serum	Neg	Neg	Neg	NT
ZIKV real-time RT-PCR urine	Neg	Pos (CT: 36.1)	Neg	NT
ZIKV real-time RT-PCR saliva	Pos (CT: 36.4)	Pos (CT: 35.4)	Neg	NT
ZIKV real-time RT-PCR semen	NT	Pos (CT: 29.6)	Pos (CT: 32.5)	Pos (CT: 30.2)
IFA ZIKV IgM titre	1:160	1:40	1:20	<1:20
IFA ZIKV IgG titre	1:640	1:1,280	1:2,560	1:640
MNT antibody titre	1:160	≥1:320	≥1:320	NT

[a] Number of days after symptom onset.

Kinetics of ZIKV RNA load measured by quantitative real-time RT-PCR in plasma, urine, and saliva samples of a patient with ZIKV infection, Italy, January 2016



Barzon L. et al. Eurosurveillance 2016

Preferred sample for diagnosis: serum or plasma

Considerations for other sample types.
In neurological cases detection of CHIKV IgM antibodies in CSF is confirmative for infection. Often IgM arise in CSF before than in serum.

Limited information is available for the isolation of virus or detection by RT-PCR in tissues or organs

- Individual cases of persistent infection in joint and muscle tissues have been reported for other alphaviruses.
- Chronic arthritis may be combined with up to 2 years persistent IgM
- In fatal cases, virus detection can be attempted on available specimens
- CHIKV RNA reported in breast milk and saliva
- Increasing evidence points at urine as possible diagnostic sample

- ✓ Campos GS et al. First Detection of Chikungunya Virus in Breast Milk. Pediatr Infect Dis J. 2017
- ✓ Musso D et al. Detection of chikungunya virus in saliva and urine. Virol J. 2016
- ✓ Bandeira AC et al. Prolonged shedding of Chikungunya virus in semen and urine: A new perspective for diagnosis and implications for transmission. IDCases. 2016
- ✓ Raut CG, et al. Utilization and Assessment of Throat Swab and Urine Specimens for Diagnosis of Chikungunya Virus Infection. Methods Mol Biol. 2016

EVD-LabNet

Modified from:
EVD-LabNet Assessment laboratory preparedness and response to chikungunya virus, updated September 2017

T cells and CHIK: role in the protection/pathogenesis

Protection

- **CD8 and innate immunity:** CD8 T cells and γδ T cells play a protective role during CHIK infection *(Wauquier N, J Infec Dis 2011, Long KM, J Virol., 2016)*
- **Treg and IL-10**: Treg cells and IL-10 associate with recovery from CHIK, suggesting that a regulatory suppressive activity is need to prevent joint inflammation *(Kulkarni SP, European Journal of Clinical Microbiology & Infectious Diseases, 2017)*

Pathogenesis

- **Inflammation:** CHIKV-induced joint damage is due to host inflammatory response mediated by macrophages, T cells, and antibodies, as well as the possible persistence of the virus in hidden sites (*Amdekar S, Viral Immunology 2017)*
- **CD4 T cells:** Transfer of CD4+ T cells from virus-infected mice into virus infected TCR-deficient mice recapitulates peak joint swelling, vascular leakage, edema, and inflammation *(Crunkhorn S, Nature review 2017).*

- **Use of immuno-suppressive drugs?**

Injection of mice with immunosuppressive drug (fingolimod) reduced joint inflammation in prophylactic and therapeutic regimens. Moreover, fingolimod limited the migration of activated and CHIKV-specific CD4+ T cells into the virus-infected joints, which reduced the severity of joint vascular leakages *(Crunkhorn S, Nature review 2017).*

Laboratory diagnosis of CHIK

- **Virological approach** (various sample types)

Virus isolation (gold standard, insect/mammalian cell lines)

Molecular tests (quali/quantitative RT-PCR, rt RT-PCR)

Sequencing (Sanger, NGS, confirmatory test)

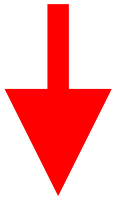
- **Serological approach** (serum/plasma)

IgM/IgG detection (ELISA or IFA)

Neutralization (confirmatory test)

Early Detection

The FASTER: We Know What It Is



Rapid Response

The FASTER: We Can Take Appropriate Action

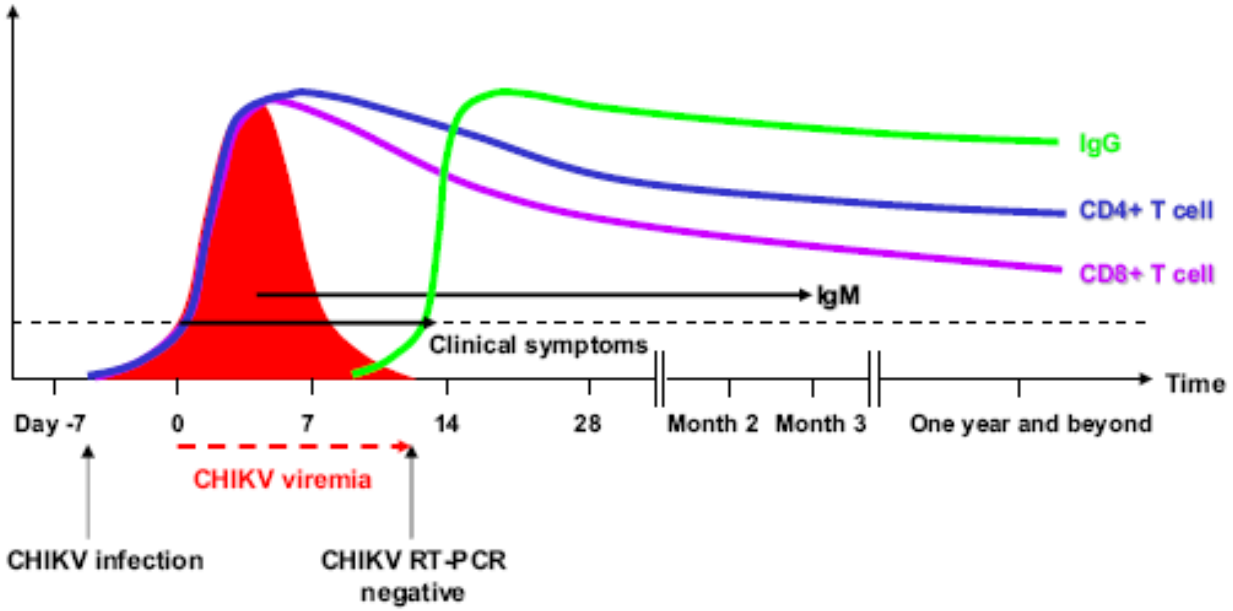
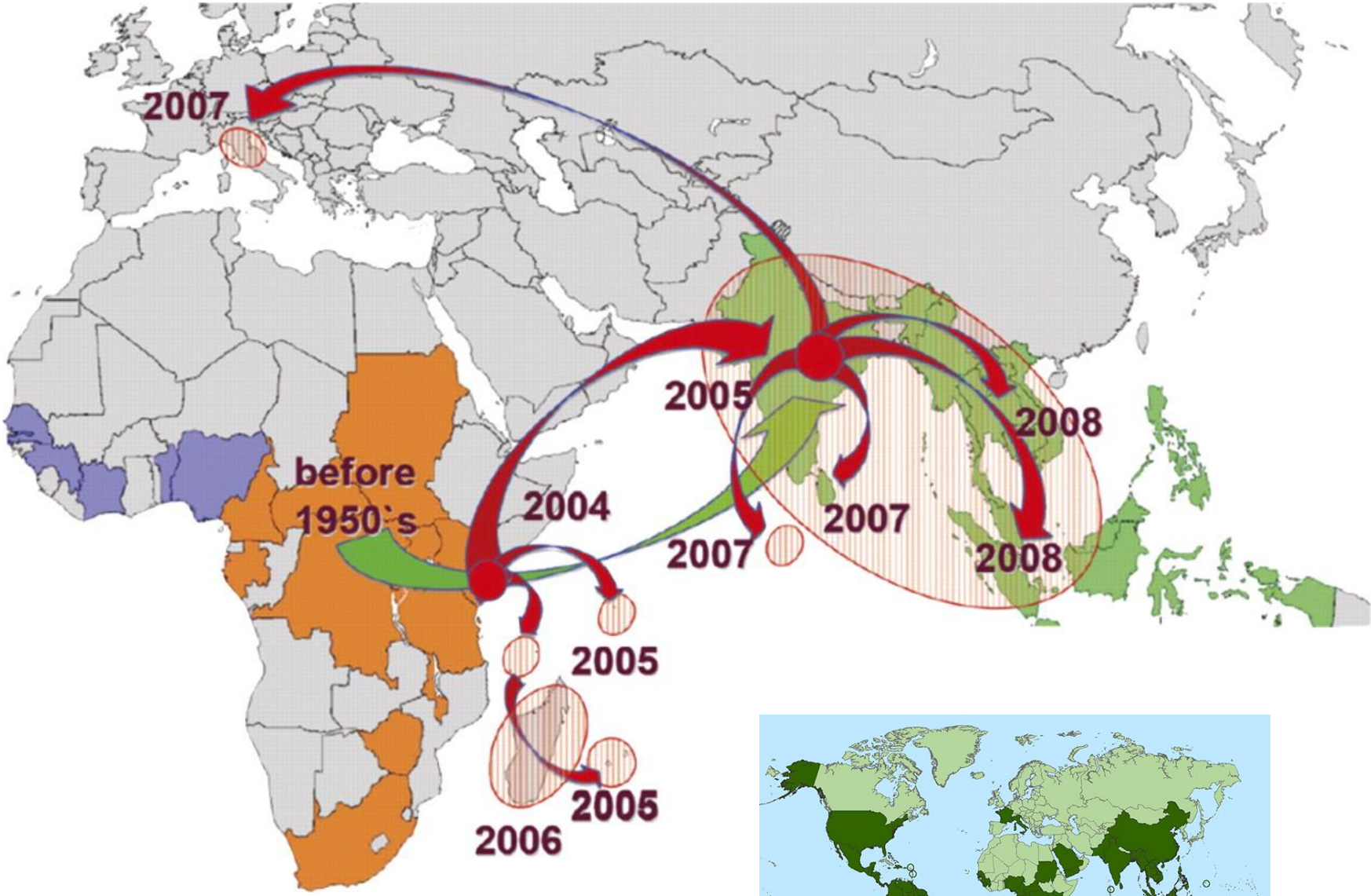


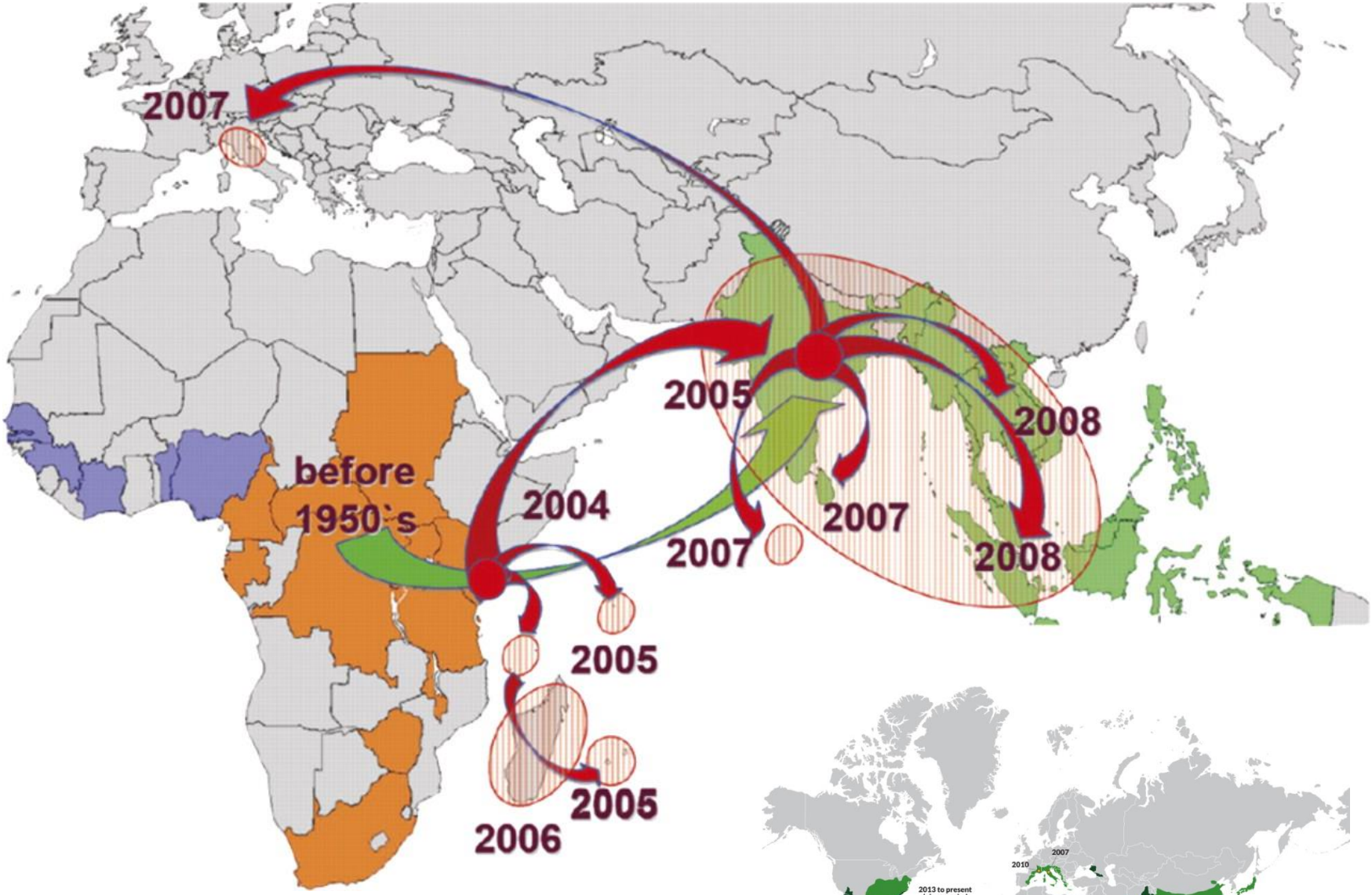
Fig. 3. Schematic representation of immune response against CHIKV infection. CHIKV viremia in blood lasts between 2 and 10 days (viral load in red filled area). Specific $CD4^+$ (blue line) and $CD8^+$ T cells (purple line) are expanded corresponding to CHIKV infection. Rapid virus elimination occurs before the expansion of neutralising antibodies (IgG, green line), Clinical symptoms (fever) disappear when CHIKV viremia drops below the level of detection. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

- Measures taken and effects monitored to control CHIKV in the affected regions are based on **confirmed CHIKV cases**

- Confirmation of presence/absence needs to be based on reliable testing (high sensitivity and specificity) and **proper sampling in time and type**


2007
2005
2008
before
1950`s
2004
2007
2007
2008
2005
2005
2006

2016
Current or previous local transmission of chikungunya virus


2007
2005
2008
before
1950`s
2004
2007
2007
2008
2005
2005
2006

2007
2010
2013 to present
(virus carried
from Far East)
pre-1958 to present
(Asian strain emerges)
2005 to present
(African strain from
Kenya mutates)
1952: virus
first identified
2014 to present
(virus carried
from Africa)
Chikungunya outbreaks
West African strain of virus
Asian strain
East/Central/South African
strain
Mosquito ranges
Areas infested with Ae. aegypti
Areas infested with Ae. albopictus
(Asian tiger mosquito)
Areas infested with both
Ae. aegypti and Ae. albopictus

Virology Journal

BioMed Central

Short report

Open Access

Chikungunya virus adapts to tiger mosquito *via* evolutionary convergence: a sign of things to come?

Xavier de Lamballerie*[1], Eric Leroy[2], Rémi N Charrel[1], Konstantin Ttsetsarkin[3], Stephen Higgs[3] and Ernest A Gould[1]

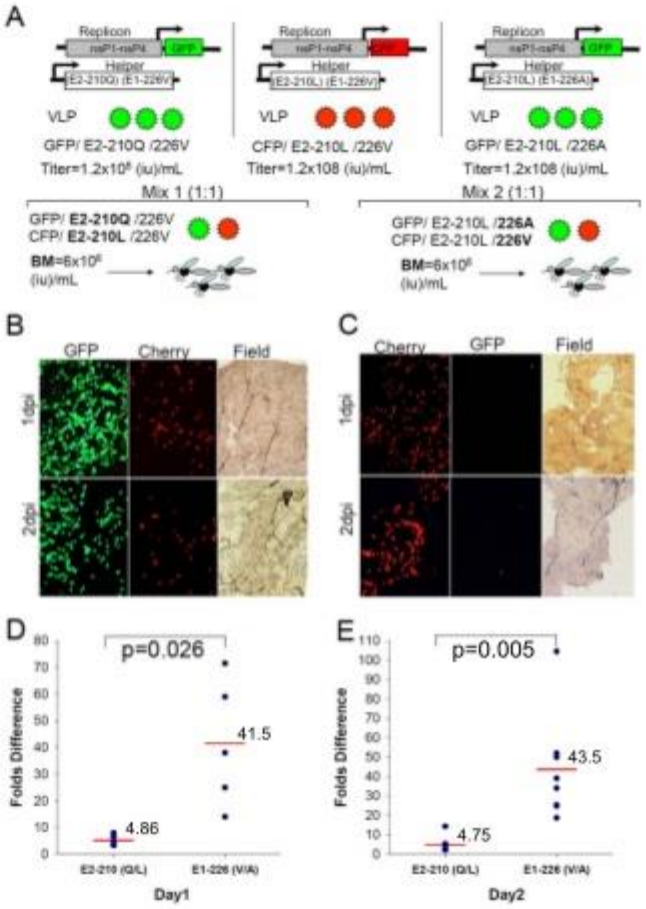
Analysis of fulllength viral sequences reveals three independent events of virus exposure to *Ae. Albopictus*, each followed by the acquisition of a single adaptive mutation providing selective advantage for transmission by this mosquito.

This disconcerting and current unique example of "evolutionary convergence" occurring in nature illustrates rapid pathogen adaptation to ecological perturbation, driven directly as a consequence of human activities.

E2 E1 substitutions enhance initial CHIKV infection of *A. albopictus* midgut cells

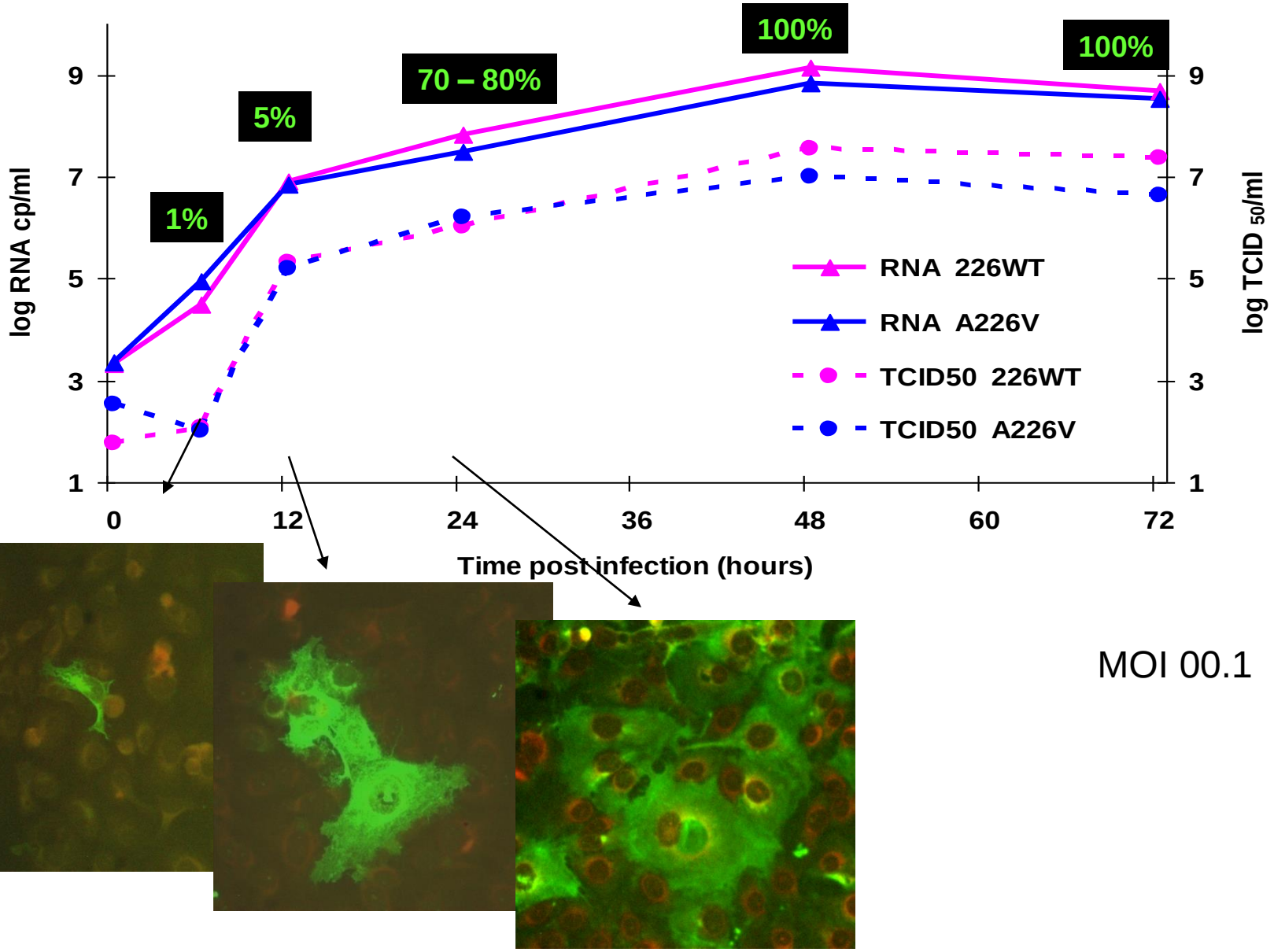
The E2-L201Q fitness increase is ca. 10-fold weaker than E1-A226V and was selected by *A. albopictus* only after ca. 4 years of circulation in India

Neither E1-A226V nor E2-L201Q affects infection of *A. aegypti*



Tsetsarkin, K.A., Weaver, S.C., 2011. PLoS Pathog 7, e1002412

Influence of A226V on CHIK replication in primate cells



MOI 00.1

La sorveglianza dell'infezione da virus chikungunya in Italia è iniziata nel 2007 e viene aggiornata annualmente dal Ministero della Salute tramite l'emanazione di un **Piano nazionale di sorveglianza e risposta**.

Generalmente in Italia, come in tutta l'Unione Europea, si registrano ogni anno sporadici casi importati in viaggiatori internazionali di ritorno da paesi infetti.
Quest'anno nel Lazio si sta verificando un'epidemia di casi autoctoni (acquisiti in Italia): le misure di sorveglianza, pertanto, sono state rafforzate sia a livello regionale che su tutto il territorio nazionale.

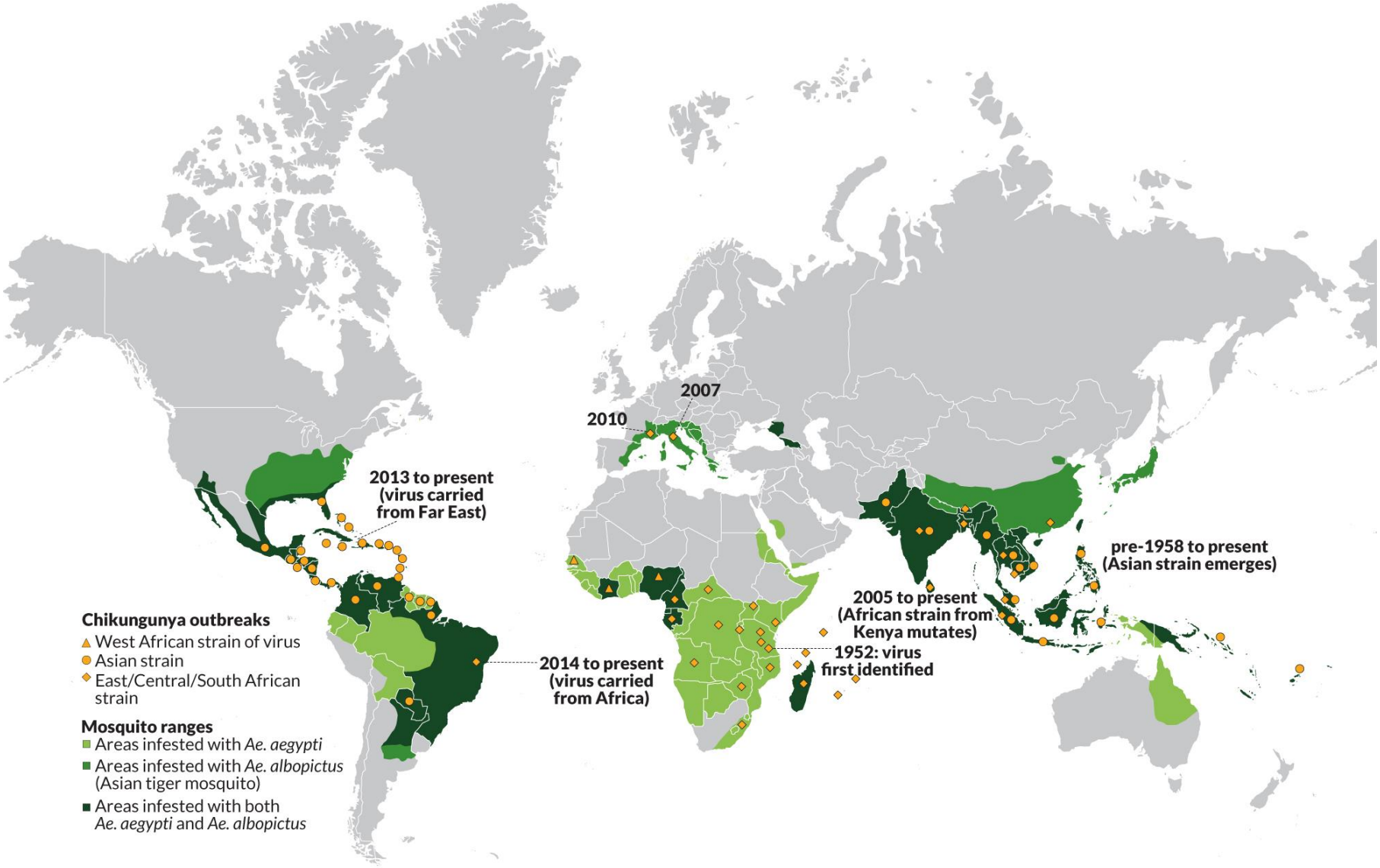
I prelievi devono essere accompagnati da:

- **All. 1 Scheda di notifica e sorveglianza**
  Circolare Regionale Nota Prot. 411678 del 08/08/2017 "Sorveglianza e controllo arbovirosi trasmesse da zanzare (*Aedes sp.*) Regione Lazio-2017

- **Allegato A2 compilato in ogni sua parte**
  - Il modello di allegato A2 specifico per gli arbovirus è accluso in calce alla procedura.

  - Tale modello è utilizzabile anche per la richiesta di esami per l'eventuale screening di donatori di organo/tessuti, qualora le disposizioni del CNT e del CNS lo prevedano.

  - **Nell'allegato A2 è necessario elencare la tipologia dei campioni inviati, il recapito telefonico del medico richiedente ed il numero di fax al quale inviare il referto**



Sources: PAHO; M. Aubry *et al*/*Emerg. Infect. Dis*. 2015; A. Powers and C. Logue/*J. Gen. Virol*. 2007; S. Weaver and M. Lecuit/*NEJM* 2015; S. Weaver/*PLoS Negl. Trop. Dis.* 2014

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LAZZARO SPALLANZANI

REGIONE
LAZIO

Istituto Nazionale per le Malattie Infettive
Struttura Complessa Laboratorio di Virologia e Laboratori di Biosicurezza
Direttore: D.ssa M.R. Capobianchi
e-mail: maria.capobianchi@inmi.it; Tel. 0655170434 Fax 065594555

Istruzioni operative per l'invio di campioni relativi alla diagnosi di infezione da virus Chikungunya al Laboratorio di Riferimento Regionale

(CHIKV Rev.0 del 09/09/2017)

Il Laboratorio di Virologia è attivo **h24**.

Il virologo di turno può essere reperito ai seguenti numeri telefonici:
0655170666
3204343793

Ulteriori recapiti utili:
Accettazione: tel 0655170674; fax 0655170676
Segreteria: fax 065594555

L'indirizzo cui inviare in campioni è il seguente
Laboratorio di Virologia
Padiglione Baglivi
Istituto Nazionale per le Malattie Infettive "L. Spallanzani"
Via Portuense 292
00149 Roma

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LAZZARO SPALLANZANI

REGIONE
LAZIO

Istituto Nazionale per le Malattie Infettive
Struttura Complessa Laboratorio di Virologia e Laboratori di Biosicurezza
Direttore: D.ssa M.R. Capobianchi
e-mail: maria.capobianchi@inmi.it; Tel. 0655170434 Fax 065594555

Istruzioni operative per l'invio di campioni relativi alla diagnosi di infezione da virus Chikungunya al Laboratorio di Riferimento Regionale

(CHIKV Rev.0 del 09/09/2017)

Fase della malattia	Tipologia di campioni	Tipologia di contenitore
Fase acuta sintomatica *(preferenzialmente entro 7 giorni, massimo entro 10 giorni dall'esordio dei sintomi)*	**- Sangue senza anticoagulanti per RT-PCR e sierologia** In base alla valutazione congiunta con il laboratorio ed alla presentazione clinica, possono essere presi in considerazione campioni aggiuntivi, **da concordare con il Laboratorio**	**- 2 Provette sterili infrangibili (almeno 4 ml ciascuna)**
Fase di risoluzione della sintomatologia *(oltre 10 giorni dall'esordio dei sintomi)*	**- Sangue senza anticoagulanti per sierologia** In base alla valutazione congiunta con il laboratorio ed alla presentazione clinica, possono essere presi in considerazione campioni aggiuntivi, **da concordare con il Laboratorio**	**- Provetta sterile infrangibile (almeno 4 ml)**

Ministero della Salute

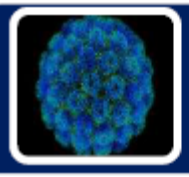
ITALY: Autochtonous Cases of Chikungunya (updated 27 October 2017)

As of 27 October, Italy had reported 331 Chikungunya cases in the Lazio region (176 confirmed and 155 probable) and 63 autochthonous confirmed cases in the city of Guardavalle, Calabria region (45 confirmed and 18 probable)

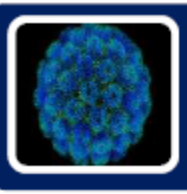
402 Total notified cases:
331 Lazio Region
63 Calabria Region
5 Emilia-Romagna Region
1 Marche Region
2 European Countries (France/Germany)

225 Total confirmed cases:
176 Lazio Region (Anzio, Roma and Latina)
45 Calabria Region (Guardavalle marina)
1 Emilia-Romagna Region with epidemiological link to Anzio
1 Marche Region with epidemiological link to Anzio
1 France with epidemiological link to Anzio
1 Germany with epidemiological link to Roma

177 Total probable cases:
155 Lazio Region (Anzio, Roma e Latina)
18 Calabria Region (Guardavalle marina)
3 Emilia-Romagna Region with epidemiological link to Guardavalle marina
1 Emilia-Romagna Region with epidemiological link to Roma



189 (**46** %) MALES
213 (**54** %) FEMALES
Median age: **55 years** (range: 0-93 ys)

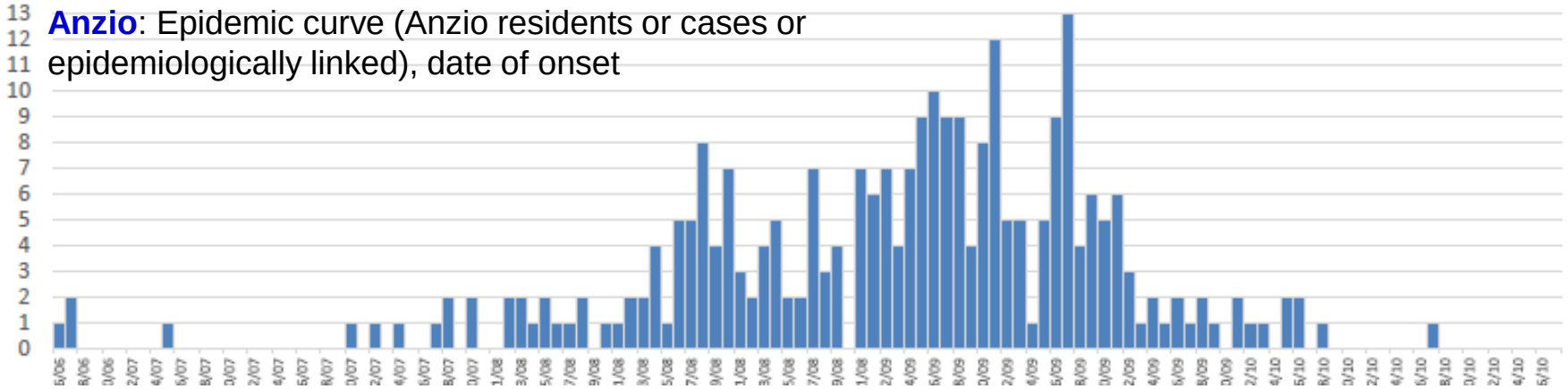


Severity of infection
Hospitalized **30** (**7** %)
Mortality: **1** confirmed

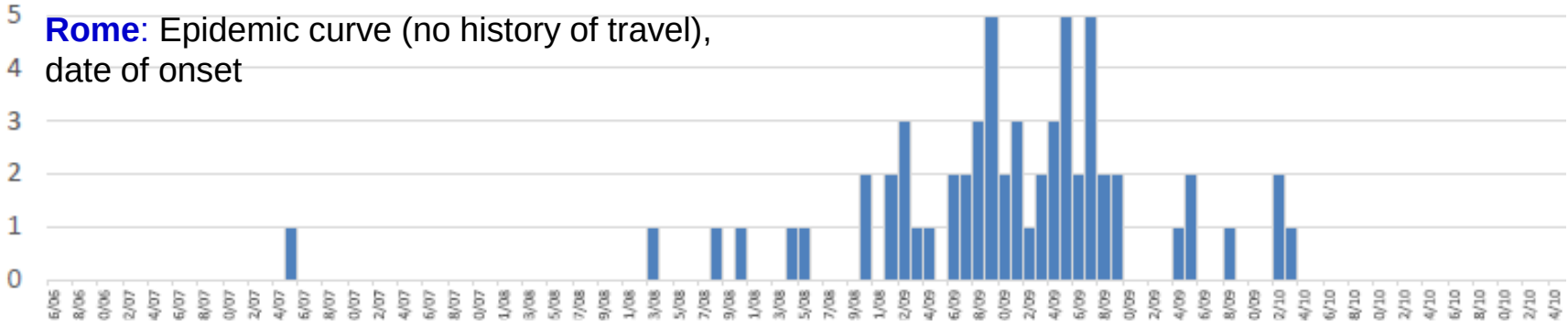
Ministero della Salute

ITALY: Autochtonous Cases of Chikungunya (updated 27 October 2017)

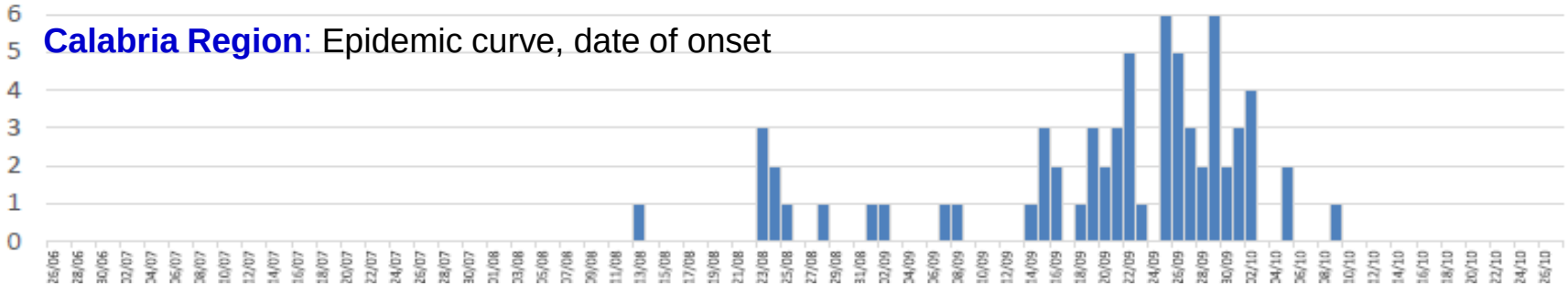
Anzio: Epidemic curve (Anzio residents or cases or epidemiologically linked), date of onset



Rome: Epidemic curve (no history of travel), date of onset



Calabria Region: Epidemic curve, date of onset

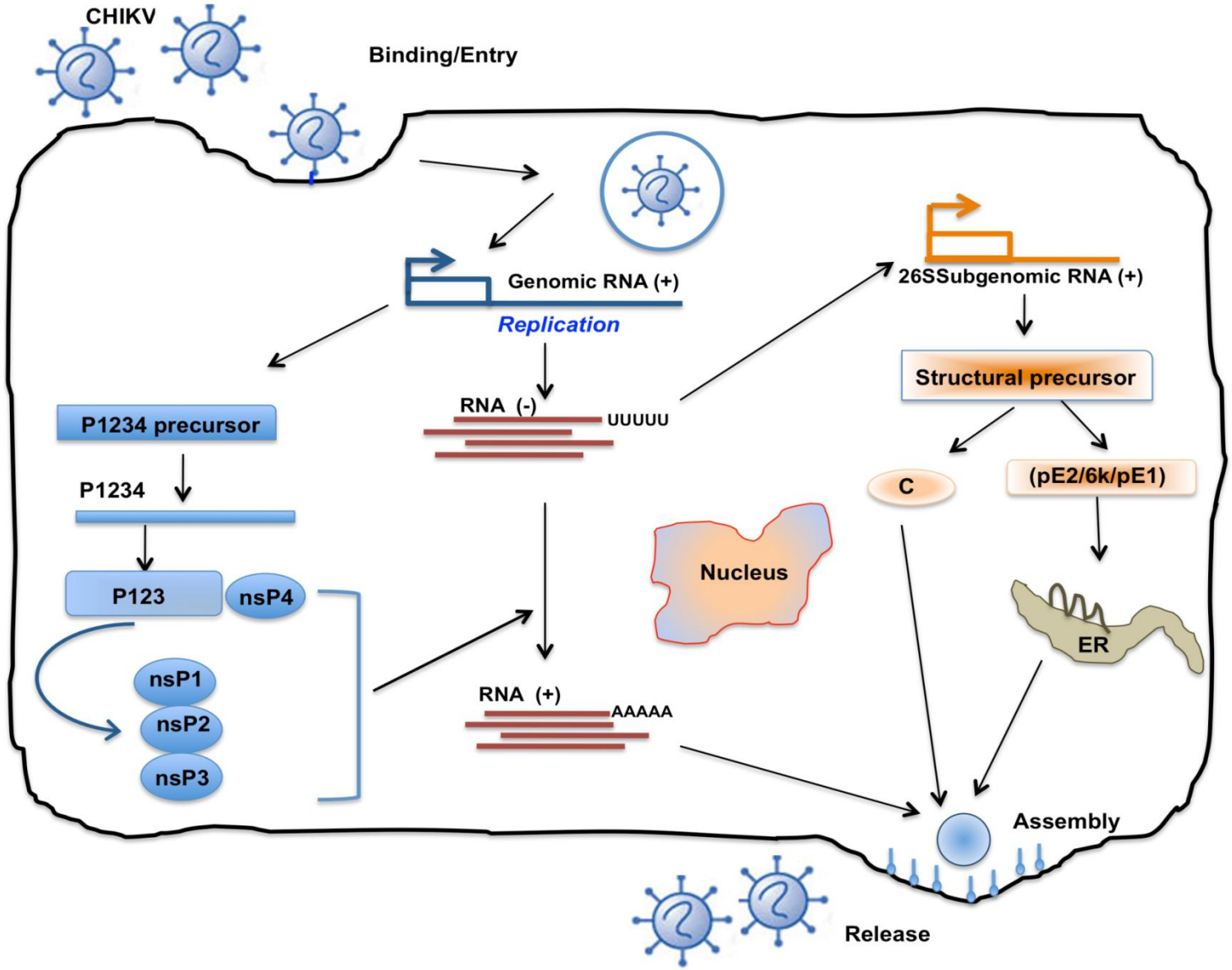


Arboviral infection diagnosis: integrated approach

- For the multiplicity of pathogens causing similar symptoms (syndromic approach), and the different kinetics of diagnostic markers, a diagnostic panel integrating multiple parameters is necessary (serology, PCR, sequencing, virus culture….)

- To overcome the general scarcity of standardized (commercial) methods, the laboratory must be integrated in networking circuits, also useful to expand the panel of available diagnostic/confirmatory tests (virtual global laboratory)

Replication cycle of chikungunya virus



Thiboutot MM et al. PLoS Negl Trop Dis 2010