Publication: Bulletin of the World Health Organization; Type: Research in emergencies
Article ID: BLT.16.171207

RECOMMENDED CITATION

Charrel RN, Leparc-Goffart I, Pas S, de Lamballerie X, Koopmans M & Reusken C. State of knowledge on Zika virus for an adequate laboratory response [Submitted]. Bull World Health Organ E-pub: 10 Feb 2016. doi: http://dx.doi.org/10.2471/BLT.16.171207

State of knowledge on Zika virus for an adequate laboratory response

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(Submitted: 10 February 2016 – Published online: 10 February 2016)

Abstract

Objective: The current epidemic with Zika virus (ZIKV) has resulted in a large increase in diagnostic requests for ZIKV in the America's but also elsewhere in travelers returning from the affected areas, especially for pregnant women with or without (past) clinical symptoms of a ZIKV infection. To support the laboratory preparedness and response for ZIKV we assessed the current state of knowledge on ZIKV diagnostics and identified present knowledge gaps for adequate ZIKV diagnostics that need to be addressed urgently.

Methods: We reviewed essential ZIKV background information and current information on ZIKV diagnostics and human ZIKV diagnosis through a literature search, using "Zika", ZIKV as search terms in PubMed. In addition we obtained information through personal communication within European preparedness networks and information provided with commercially available diagnostic tests.

Findings: Essential knowledge on geographic spread, pathogen characteristics, life-cycle, infection kinetics, available tests for molecular-based and serology-based diagnosis and biosafety issues are reviewed. Specifics of tests used for human ZIKV diagnosis are discussed in the context of the current outbreak strain.

Conclusion: Besides provision of essential ZIKV knowledge to the world laboratory community, knowledge gaps have been identified that need to be addressed urgently.

- Rapid and extensive field validation of available molecular and serology tests in background of both affected areas and areas unaffected but welcoming returning travellers. Special focus should be given to the patient population of pregnant women.
- Monitoring of genomic diversity of circulating ZIKV strains to allow verification against operational molecular tests to ensure continuous sensitivity.
- Development of External Quality Assessments (EQA) for both molecular and serology testing for multiple sample types.
- Insight in infection kinetics should be obtained through prospective studies, including pregnant women as well, to determine ideal sampling type or combinations thereof and sampling time. This should focus on plasma/serum, urine and saliva combinations.
- The availability of reagents for diagnostic development should be facilitated.

Background

On 1 February 2016, the World Health organization (WHO) declared that the recent cluster of microcephaly cases and other neurological disorders reported in the America's, where an outbreak with Zika virus (ZIKV) is ongoing, constitutes a Public Health Emergency of International Concern (PHEIC) (1).

Zika virus (ZIKV) is a mosquito-borne virus (genus *Flavivirus,* family *Flaviviridae*) related to yellow fever, dengue, West Nile, Japanese encephalitis and tick-borne encephalitis viruses. ZIKV was first isolated in 1947 from Rhesus macaques living in the eponymous forest in Uganda (2). Up to 2006, only sporadic cases of ZIKV human infections were reported in literature (3). Accordingly, ZIKV was long considered a low-impact human pathogen, which might explain the limited literature (only 269 references in NCBI PubMed in January 2016), compared to other mosquito-borne viruses such as dengue virus (9187 references), West Nile virus (5949 references) or chikungunya virus (2183 references)(4).

ZIKV infection is unapparent in approximately 80% of the cases as observed during the Micronesia and French Polynesia epidemics in 2007 and 2013-2014, respectively (5, 6). Incubation can range from 3 to 12 days. Common symptomatic forms are characterized by a macular/ papular rash (90-96%), fever (62-65%); myalgia and arthralgia (48-65%), headache (45-58%), non-purulent conjunctivitis (38-55%) and retro-orbital pain (40%) (5, 7, 8).

The current major concerns, besides globalization and an expected huge number of cases including an increase of exported ZIKV cases (Table 1), are the possible association with Guillain-Barré syndrome (GBS), and microcephaly and other neurological manifestations in new-borns in the current epidemic region. Both noticed and identified retrospectively in French Polynesia respectively (8-11). Although the link between ZIKV and GBS or microcephaly still needs to be established unequivocally, the question arises whether the increased incidences of

GBS and microcephaly in the current outbreak region are due to a specific virulence of certain viral strains or a common pattern of all ZIKV strains that went unnoticed because of the lower number of cases in previous outbreaks (8, 12). In Brazil, more than 4,700 cases of suspected microcephaly have been recorded from mid-2015 to end January 2016, where the usual number is consistently below 200 cases per year (13), while Brazil, Colombia, Surinam, Venezuela and El Salvador have reported spikes in GBS cases in January 2016 (10, 14-18) . While it remains to be determined if ZIKV infection causes these complications, several governments and health agencies have issued a travel warning for the affected region, with specific attention to pregnant women, as a precautionary measure (19-21).

The current epidemic with ZIKV has resulted in a large increase in diagnostic requests for ZIKV in the America's but also in returning travellers from the affected areas, especially for pregnant women with or without (past) clinical symptoms of a ZIKV infection. Therefore, the preparedness for ZIKV in both affected and unaffected regions needs an assessment of the current situation from the laboratory perspective to ensure an adequate (timely, accurate) laboratory response. Here, the essential knowledge and knowledge gaps are reviewed and discussed.

Table 1. Overview of traveller related import cases of Zika virus.

Imported in	Imported from	# of human cases	Zika virus RNA detection results	Zika virus Serology results*	reference
Australia	Cook islands	1	Serum pos	IgM+SC(IgG)+SC(IgM)	(81)
Australia	Indonesia	1	Serum pos	-	(95)
Canada	Thailand	1	Serum, urine pos	IgM+SCNT	(59)
Finland	Maldives	1	Urine pos	Not tested	(77)
Germany	Thailand	1	Serum neg	IgM + IgG	(67)
Germany	Indonesia	1	Serum neg	IgM+SC (IgM/IgG)+NT	(96)
Italy	Brazil	1	-	IgM+SC(IgG)+SCNT	(85)
Italy	French Polynesia	2	Serum pos	SC(IgM/IgG)	(84)
Japan	Thailand	1	Serum equiv, urine pos	IgM	(58)
Japan	French Polynesia	2	Serum, urine pos	IgM+SCNT	(97)
Norway	French Polynesia	1	Serum pos	SC(IgM/IgG)	(98)
United States	French Polynesia	1	-	IgM+SC(IgG)	(99)

*SC, seroconversion; NT, neutralization assay; SCNT, seroconversion observed using NT; equiv, equivocal (discrepant results observed with the two assays; pos = positive; neg=negative

Knowledge and knowledge gaps

Geographic spread.

Since its first isolation in 1947 in Uganda, serological, epidemiological and entomological studies reported the circulation of the ZIKV in tropical areas of western Africa (Nigeria, Sierra Leone Ivory Coast, Cameroon and Senegal), central Africa (Gabon, Uganda and Central African Republic), and in Asia (Pakistan, Indonesia, Philippines, Malaysia, Cambodia and Thailand) (22-24). Since 2007, the virus caused outbreaks in several islands of the pacific region (Micronesia, Cook Islands, French Polynesia, New Caledonia, Guam, Samoa, Vanuatu and Solomon Islands). It is estimated that approximately 30,000 cases occurred in French Polynesia during the 2007 outbreak, thus infecting 11.5% of the population of 260,000 (10, 11).

Although seroprevalence studies conducted in Africa (25-29) and Asia (30) suggest that epidemics of ZIKV infections have occurred in Africa already before the 2007 Yap outbreak, only sporadic cases of ZIKV infection were described. Ever since, ZIKV has been reclassified as an re-emerging virus which has proven its capacity to spread to the Pacific. From October 2015 to January 2016, approximately 7,000 cases were recorded on the Cap Verde islands; whether this outbreak is due to a viral strain belonging to the African or to the Asian lineage remains unknown at this time (31).

In May 2015, the public health authorities of Brazil confirmed autochthonous transmission of ZIKV in its North-eastern states, the first known emergence of this virus in mainland South-America (32, 33). As of February 5, 2016 ZIKV autochthonous transmission has been reported in 26 countries and EU outermost regions in the Americas (Barbados, Bolivia, Brazil, Colombia, Costa Rica, Curacao, Dominican Republic, Ecuador, El Salvador, French Guiana, Guadeloupe, Guatemala, Guyana, Haiti, Honduras, Jamaica, Martinique, Mexico, Nicaragua, Panama,

Paraguay, Puerto Rico, Saint Martin, Suriname, US Virgin Island and Venezuela) (18, 34). The increased import of travel-associated ZIKV to Europe is illustrated by recent reports of imported cases in Austria, Denmark, Finland, France Germany, Ireland, Italy, Portugal, the Netherlands, Spain, Sweden, Switzerland and the UK (35).

Pathogen, vector transmission and alternative transmission pathways.

Virions of ZIKV are 40–60 nm in diameter, spherical in shape and contain a lipid envelope. Its genome consists of a positive sense RNA of approximately 11 kb. The virions consist of a single capsid (C) and two membrane-associated envelope proteins (M, E). The nonstructural proteins (NS1-NS5) contain sequence motifs characteristic of a serine protease, RNA helicase and RdRp (NS5). The genomic RNA contains a single long ORF flanked by 5'- and 3'-terminal non-coding regions (NCRs) that form specific secondary structures required for genome replication and translation. Translation-initiation of genomic RNA is cap-dependent. Viral proteins are synthesized as part of a polyprotein that is co- and post-translationally cleaved by viral and cellular proteases. RNA synthesis occurs in the cytoplasm in association with modified cellular membranes via synthesis of full-length negative-strand intermediates. Virion assembly, including acquisition of the glycoprotein-containing lipid envelope, occurs by budding through intracellular membranes. Viral particles are transported in cytoplasmic vesicles through the secretory pathway before they are released by exocytosis (36, 37).

ZIKV belongs to the Spondweni virus serogroup of mosquito-borne viruses in the flavivirus genus (Figure 1a). Phylogenies reveal the existence of 2 lineages (Figure 1b): the African lineage which has showed no propensity to disseminate outside of Africa, and the Asian lineage which continues to seed in previously unaffected regions of the world (38, 39). All strains having

recently disseminated belong to the Asian lineage (with Cape Verde outbreak strain of unknown lineage) (32, 33, 40, 41). ZIKV genomes from patients infected in Surinam and Brazil in 2015 are closely related to the strain that circulated in French Polynesia in 2013, with more than 99·7% and 99·9% of nucleotide and amino acid identity, respectively (41)(Figure 1).

Zika virus is transmitted by *Aedes* mosquitoes and *Ae. aegypti* is the only species for which transmission outside Africa has been confirmed. In the 2007 Yap island outbreak, *Ae. hensilii* mosquitoes were implied as vector, but this could never be confirmed by virus detection. The virus has been isolated and/or detected by PCR from *Ae. africanus*, *Ae. aegypti*, *Ae. albopictus*, *Ae. apicoargenteus*, *Ae. luteocephalus*, *Ae. vitattus, Ae. taylori*, *Ae. dalzieli*, *Ae. hirsutus, Ae. metallicus, Ae. unilinaetus,* Ae. opok and *Ae. furcifer* species in the field in Africa (3, 24, 42). In addition ZIKV genomic RNA was detected in *Mansonia. uniformis, Culex perfuscus* and *Anopheles coustani* mosquitoes in Senegal (24). *Ae. albopictus* has shown competence for ZIKV dissemination in laboratory circumstances but has never been implied in ZIKV epidemiology in the field outside Africa (42, 43).

Additional modes of transmission have been identified. Perinatal transmission can occur most probably by trans-placental transmission or during delivery when the mother is infected (44, 45). ZIKV has been isolated from semen collected 14 days post start of symptoms (46) while detection of ZIKV genomes was described in semen 28 days post onset of symptoms (47). Sexual transmission was indicated in three case reports (46, 48, 49). There is a potential risk of ZIKV transfusion-derived transmission (50, 51) and Brazilian authorities announced the first cases of blood-transfusion mediated transmission on 5 February, 2016 (52).

Infection kinetics.

Knowledge of ZIKV infection kinetics is essential to determine the optimal strategy of diagnosis. Only few cases of patient diagnosis for ZIKV (all Asian lineage) are described in the literature.

Viremia and presence of virus in other fluids.

Data from French Polynesia show that viremia is low and short-termed (53-55). ZIKV can be detected in serum typically up to 3-5 days after the clinical onset; the viral load seems to peak when clinical signs appear (33, 54, 56). ZIKV can be detected in saliva but not longer than in serum. The combination of blood and saliva increased the rate of detection: 19% of 182 cases were detected via saliva only (53). Data observed in six patients in New Caledonia and other case reports, showed that detection of ZIKV in urine should be combined with blood samples for molecular testing (54, 57, 58) . In urine the viral load appeared higher than in blood with a peak between days 5-7, and seemed to last longer with PCR detection up to 20 days after clinical onset (54). In one patient from Suriname ZIKV RNA was still detected in urine at day 28 post onset of illness (*own observation*). Virus isolation from urine in Vero cells has been described (59). Twice detection of ZIKV RNA in nasopharyngeal swabs was described while serum was negative (59, 60). The reduced invasiveness of urine and saliva collection is an advantage for diagnosis of infants and new-borns. More studies are needed as these observations are based on a limited number of cases.

Immune response.

Immune response during Zika virus infection was only described for a small number of patients (n=11) during the ZIKV virus outbreak in Yap (56). By using MACELISA for IgM and capture ELISA for IgG with whole viral antigen (inactivated virus) and monoclonal antibodies, IgM was found to appear as soon as 3 days after the onset of symptoms. IgG seemed to appear after day

10 in a patient with no history of previous flavivirus infections (56, 61, 62). In this patient, neutralizing antibodies against ZIKV could be detected as early as 5 days after the onset of fever. The current ZIKV outbreak reveals a special group of patients, pregnant women. As antibody responses during pregnancy may be different from those in non-pregnant individuals (63, 64) specific attention should be given in prospective studies to determine the ZIKV immune responses in pregnant women.

Typically for flaviviruses, IgM antibodies develop within a few days after onset of illness and can generally be detected up to three months. IgG antibodies develop within days after IgM and can be detected for months to years. Cases have been described with persistence of IgM antibodies for longer periods which complicates accurate diagnostics (65).

Molecular diagnosis.

Molecular diagnosis of ZIKV can be done on different types of body fluids: serum, EDTA-plasma, saliva and urine. Urine and saliva should be considered together with blood/serum in the algorithm of ZIKV genome detection using molecular techniques (see above). The reliance on the use of molecular diagnostics to rule out infection requires careful consideration, as experience with clinicians and diagnostic laboratories is limited by default for emerging diseases. A number of in-house RT-PCR tests for ZIKV have been described in literature but very few provide validation using the recent viral strains and fully documented clinical specimens. Here, we only discuss RT-PCR assays that have resulted in the detection of viral RNA in at least one human ZIKV case described in a peer-reviewed article indexed in NCBI PubMed or by pers. comm. (Table 2). We have mapped (mis)-matches of the primers/ probes used in these assays with the currently known ZIKV sequences (Figure 2).

Table 2. Overview RT-PCR methods and sample types used to detect human Zika virus cases.

PCR	Reference PCR	PCR Target	PCR technique	Amplicon size (bp)	ZIKV lineage analytical	ZIKV lineage field	# human patients	Sample types pos in field	Reference
ZIKV-specific									
Lanciotti 2008 set 1 [835]	(56)	ZIKV-NS5	Hydrolysis probe	76	Asian, African	Asian	>200 (combined set)	serum, urine, amniotic fluid	(5, 9, 44, 46, 50, 53, 54, 55, 58, 96, 99, 100)
Lanciotti 2008 set 2 [1086]	(56)	ZIKV-NS5	Hydrolysis probe	76	Asian, African	Asian	>200 (combined set)	serum, urine, amniotic fluid	(5, 9, 44, 46, 50, 53, 54, 55, 58, 96, 99, 100)
Faye 2013	(66)	ZIKV-NS5	LNA-probe	102	Asian, African	African	0	serum	Rockx, *pers. comm* .
Tappe 2014	(67)	ZIKV-NS3	Hydrolysis probe	94	Asian	Asian	5	serum	(67, 84,85, 95, 97)
Faye 2008	(68)	ZIKV-E	conventional	364	African	Asian	>15	serum	(32,33)
Pyke 2014	(81)	ZIKV-NS1	Hydrolysis probe	65	Asian	Asian	1	serum	(81)
Pyke 2014	(81)	ZIKV-E	Hydrolysis probe	71	Asian	Asian	1	serum	(81)
Pan-flavi with sequencing									
Moureau 2007	(72)	Flavi-NS5	Sybr-green based	269-272	African	Asian	2	serum, urine	(57, 78)
Kuno 1998	(73)	Flavi-NS5	conventional	1079	Asian, African	Asian	51	serum	(79)
Scaramozzino 2001	(74)	Flavi-NS5	conventional (seminested)	220	African	Asian	1	serum, urine	(81), Barzon *pers. comm.*
Maher-sturgess	(76)	Flavi-NS5	conventional	800	African	Asian	1	serum	(80)
Ayers 2006	(75)	Flavi-NS5	conventional	863	nt	Asian	1	serum, urine, nasopharyngeal	(59)

ZIKV-specific reverse transcriptase PCR.

To date multiple real-time and conventional RT-PCRs specific for ZIKV have been described (Table 2) (56), (66-68). The combined assays of (56) are the most commonly used in literature for direct diagnosis of ZIKV (5, 50, 55, 69, 70). Lanciotti (56) described two targets, and mentioned equivocal positive results which could be related to false positives. Of 157 samples tested, 10 samples were positive for only one target while 17 samples were positive for both. It was not mentioned whether this was randomly observed with both assays. However, discrepant results were observed in the French and Dutch reference laboratories as well. Set 1 in combination with fast virus master mix (FVMM, Lifetech) was found to be less sensitive than Set 2 (~ 3 Ct) and showed worse amplification plots with ZIKV African lineage. For set 2 African and Asian lineages had comparable sensitivity and amplification plots. Positivity with only set 1 was shown to be due to false positives rather than a lack of sensitivity of Set 2 by sequencing. Recently, this assay was used to detect ZIKV RNA in amniotic fluid of two pregnant women from the state of Paraiba, Brazil who were diagnosed with fetal microcephaly (69).
Faye (66) relies on a LNA-probe. Unlike indicated in the article, the probe used should be FAM - CTYAGACCA**GCTG**AAG – BBQ, with the LNA nts in bold (M. Weidmann, *pers. comm.)*. In literature this assay has not been used yet for the diagnosis of Zika virus infection in humans but several laboratories are currently identifying human ZIKV cases using this assay (24, 66) (B. Rockx *pers. comm*.). In general, LNA and MGB probes are not the system of choice to be used in screening PCRs, since only one mutation can result in false negative results due to detection failure of the amplified product. In cases like Zika virus where only a minimum of sequence data is available and the grade of genome conservation is not known, it is generally recommended the

use of TaqMan probes in real-time PCR. Balm et al (71) describe a ZIKV specific RT-PCR that so far has not been linked to detection of human cases in literature.

Pan-flavivirus RT-PCR in combination with sequencing.

One real-time (72) and multiple conventional Pan-flavi RT-PCRs (73-76) have been used in combination with sequencing to detect human ZIKV cases (57, 59, 77-80) (Table 2).

Commercial tests.

Commercial assays for ZIKV become rapidly available. However as for now the tests are for research purposes only. Usually, primers and probe sequences in commercial kits are not publicly available precluding *in silico* assessment of the fit with the current ZIKV. Known commercial kits recently put on the market are RealStar® Zika Virus RT-PCR Kit 1.0 (Altona, Germany), Genesig® Advanced Kit (Genesig, UK), MyBioSource Zika PCR Kit (USA), Genekam Zika virus PCR, (USA) and a ZIKV PCR kit by Fast-Track (Luxembourg) . Companies should be encouraged to put detailed information on their primers/probes in the public domain so their performance can be evaluated continuously against the evolving genomic diversity of ZIKV during the outbreak. The Altona PCR kit is CE/IVD-certified as of February 2016.

Serology

Flavivirus serology is complex due to extensive cross-reactivity between antibodies triggered by different flavivirus infections or vaccination, even for viruses belonging to different serogroups. In addition, an acute flavivirus infection might boost cross-reactive antibodies due to (previous) flavivirus infection or vaccination (65). Different viruses and serology outcomes should be taken into account in different contexts (Table 3). Patients in the current ZIKV outbreak area will have a high level of flavivirus background, *viz.* DENV, YFV, WNV (Table 2), while returning European travellers will not. As a consequence, a high proportion of the ZIKV infections in the outbreak region will be secondary flavivirus infections and thus complicate serology. Although some cross-reactivity can still occur (see below), the most specific serology test for flaviviruses are virus neutralization tests. A very limited number of serology tests have been described in literature.

Published data from 1950 to 1980 on ZIKV seroprevalence and diagnosis studies show the use of complement fixation (CF), haemagglutination inhibition (HI) and neutralization tests. CF and HI tests show extensive cross reaction with other flaviviruses (26, 81, 82). More recent publications describe in-house developed ZIKV serology based on ELISA using whole viral antigen or recombinant protein, or immunofluorescence (IFA). These tests have been only marginally validated and the laboratory community is in an urgent need for validation of serology tests in the field.

In-house tests

Immunoglobulin M antibody-capture enzyme-linked immunosorbent assay (MAC-ELISA) and capture ELISA for IgG using whole inactivated viral antigen produced on suckling mouse brains

have been used to map antibody responses in 11 patients from the Yap outbreak in 2007 (56). A similar technique using whole viral antigen produced in cells, was used to describe the antibody response of a GBS patient (9). MAC-ELISA in a primary ZIKV infection with no history of other flavivirus infection or vaccination, seems to be quite specific for ZIKV with no cross-reaction with other flaviviruses (56) (Leparc-Goffart, 2016 unpublished data). A case of ZIKV virus infection in Australia imported from Cook Island was diagnosed by RT-PCR but also with a seroconversion for Zika IgM and IgG between an acute sample at day 2 after the onset of symptoms and a convalescent sample at day 10 using an in-house ZIKV IgM and IgG typing microsphere immunoassay using recombinant NS1 (80). Diagnosis of imported cases in Europe were described in different publications by using whole virus immunofluorescence assays (IFA) for ZIKV IgM and IgG to determine seroconversion or a fourfold titre increase between acute and convalescent serum samples (67, 83, 84). Virus neutralisation tests have been used in a few papers to confirm antibody responses detected by ELISA or IFA (9, 56, 83). In a patient with a primary flavivirus infection for ZIKV, comparative neutralization tests only showed neutralizing antibodies against ZIKV and not against DENV1-4. The interpretation of neutralization tests when a patient has already been infected by another flaviviruses (or vaccinated) is more complex. Even if the neutralization titre was higher for ZIKV than for other flaviviruses, only a very few patients had a titre for ZIKV a fourfold higher than for other heterologous flaviviruses (56).

	Population							
	(European) travelers	**Europe**	**South America**	**Central America and the Caribbean**	**North America**	**Africa**	**Asia**	**Oceania**
Vaccine								
YFV	x		x			x		
JEV	x						x	x
TBEV	x	x						
DENV				x*				
Endemic circulation								
YFV			X[1]			x		
DENV			X[1]	X[1]		x	X[1]	x
WNV		x	x	x	x	x	X[1]	x
JEV							X[1]	x
TBEV		x					x	
USUV		x				x		

SLEV	x	x	x		
ROCV	x				
ILHV	x				
MVEV					X[1]
KFDV				x	
AHFV				x**	
WSLV			x		

Table 3. Overview putative endemic circulating or vaccine-related cross-reacting infections with flaviruses other than Zika virus.

[1]Highest priority based on (65). * only in Mexico and possibly Brazil in near future (H. Zeller *pers. comm.)* **Only in Middle-East region.

YFV = yellow fever virus; JEV = Japanese encephalitis virus; TBEV = tick-borne encephalitis virus; DENV = dengue virus; WNV = West Nile virus; USUV= Usutu virus; SLEV = St Louis encephalitis virus; ROCV = Roccio virus; ILHV = Ilheus virus; MVEV = Murray valley encephalitis virus; KFDV = Kyasanur forest disease virus; AHFV = Alkhumra haemorrhagic fever virus; WSLV = Wesselsbron virus.

Commercial tests.

Commercial serology tests will become increasingly available. To our knowledge three commercial tests are on the market or will be available soon. The Zika Virus IgG (ZV-IgG) or IgM, ELISA Kits by MyBiosource (USA) use a double-antigen sandwich ELISA. No information on the type of antigens used or on the validation specifics to determine cross-reactivity is given (85, 86). Biocan Diagnostics (Canada) offers a rapid finger-prick assay based on a mix of ZIKV NS1 and Envelope protein, that can detect IgM and IgG antibodies. The company indicates a specificity of 99% but no specifics of the validation procedure are given (87). Euroimmun (Germany) offers an IgM/IgG IFA an IgM/IgG ELISA based on NS1 protein. The ZIKV IFA is offered in a mosaic slide, together with DENV1-4 and chikungunya virus. Provided information indicate cross-reactivity with antibodies directed against TBEV, WNV and DENV for both IgG and IgM assay. Furthermore, validation data indicate for the IgM and IgG ZIKV IFA a wide range of specificities and sensitivities depending on the validation cohort. The given values are hard to interpret as the description of the cohorts is too brief. Furthermore, these data should be interpreted with great caution as positivity was only rated in the cut-off dilution. This could mean that the specificity can be different than given (higher) as the results were not scored as end-titers. The use of end-titers would provide a window for differentiating the (cross) reactivity measured. The Euroimmun ZIKV ELISA is based on recombinant NS1 protein which leads to a reduction of cross-reactivity with other flaviviral antibodies to maximal values of 18.8% (IgG) and 8.3% (IgM). Euroimmun seems the only manufacturer actually providing detailed validation data and their data clearly address and illustrate the above-mentioned difficulties with cross-reactivity in flavivirus sero-diagnostics (88).

All tests are for research purposes only and none are CE-certified at this moment.

Biosafety

Zika virus is a Biosafety level 2 pathogen in the EU (with exception of the UK) and USA. There are no inactivation data specific for ZIKV available. However typically, flaviviruses are inactivated by temperatures above 56 °C for at least 30 minutes, pH ≤6, UV-light and gamma-radiation and are known to be susceptible to disinfectants like 1% sodium hypochlorite, 2% glutaraldehyde, 70% ethanol, 3-6% hydrogen peroxide and 3-8% formaldehyde (89-92). Flaviviruses are susceptible to phenol guanidine isothiocyanate (e.g. TRIzol ®) and chaotropic salts (e.g. AVL buffer ®) (89, 93). Although ZIKV is a BSL2 pathogen, laboratories should assess the risks for pregnant laboratory personnel, especially when the virus is cultured (e.g virus neutralization tests).

Synthesis knowledge gaps and conclusions.

Knowledge gaps for an adequate ZIKV laboratory response have been identified and need to be addressed urgently. This should be achieved by a WHO coordinated international laboratory response including a study protocol for prospective studies addressing the most pressing information needs. Obtained knowledge should be put in the public domain as soon as possible.

- Rapid and extensive field validation of available molecular and serology tests in background of both affected areas and areas unaffected but welcoming returning travellers. Special focus should be given to the patient population of pregnant women.
- Monitoring of genomic diversity of circulating ZIKV strains to allow verification against operational molecular tests to ensure continuous sensitivity.
- Development of External Quality Assessments (EQA) for both molecular and serology testing. Besides ZIKV positive samples these should include well-defined materials positive for viruses or antibodies of a cross-reactive panel (DENV, YFV, WNV) and a

panel with similar clinical manifestation (chikungunya virus, malaria, rickettsia). In addition the EQA for molecular testing should address different types of samples; serum, urine and saliva.

- Insight in infection kinetics should be obtained through prospective studies, including pregnant women as well, to determine ideal sampling type or combinations thereof and sampling time. This should focus on plasma/serum, urine and saliva combinations.
- The availability of reagents for diagnostic development should be facilitated, e.g. virus strains, virus antigens, quantified viral RNA for instance through the EU consortium EVAg and near-future ECDC labnet.

Funding

This work received funding from the European Union Seventh Framework Programme (FP7) under (i) the project PREPARE (grant agreement No 602525) (CR, SP,MK), (ii) the European Virus Archive goes Global (EVAg) project that has received funding from the European Union's Horizon 2020 research and innovation program under grant agreement No 653316 (RC, ILG, XdL, CR, SP, MK), (iii) under the project PREDEMICS FP7/2007-2013 – n°278433 (http://predemics.biomedtrain.eu/cms/)(RNC, ILG, XdL), (iv) the EDENext FP7- n°261504 EU project and this paper is catalogued by the EDENext Steering Committee as EDENext xxx (http://www.edenext.eu) (RNC, ILG, XdL). The work of RNC was done under the frame of EurNegVec COST Action TD1303.

Competing interests: None declared

Ethical approval: Not applicable.

Acknowledgements: We wish to thank Luisa Barzon and Barry Rockx for sharing information on ZIKV diagnostics in their laboratories and Hervé Zeller for critical reading of the manuscript..

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Figure 1. Phylogenetic relationships within family Flaviviridae (A) and between Zika virus strains belonging to the two lineages (B) based on complete genomic sequence analysis (Neighbour Joining).

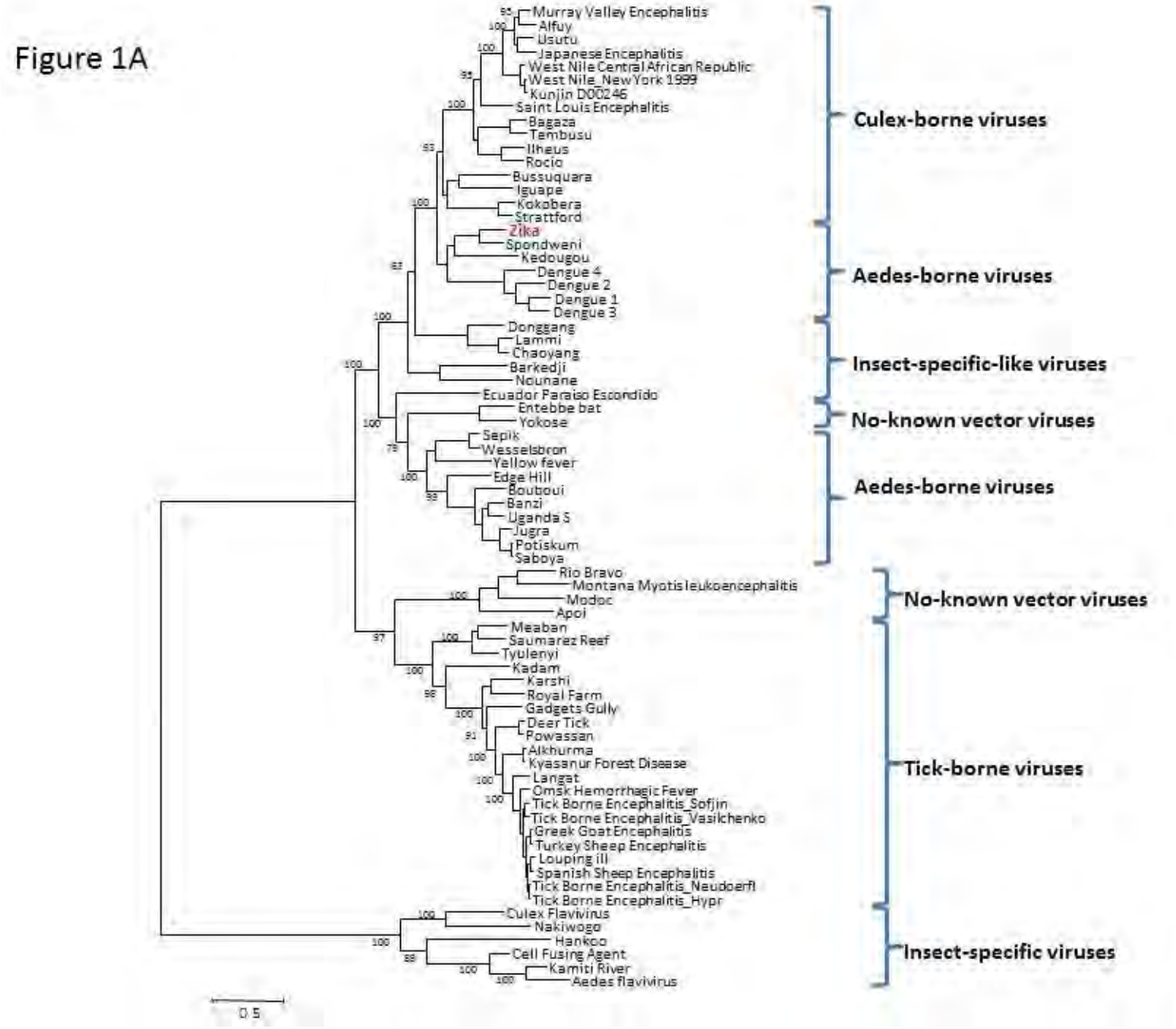
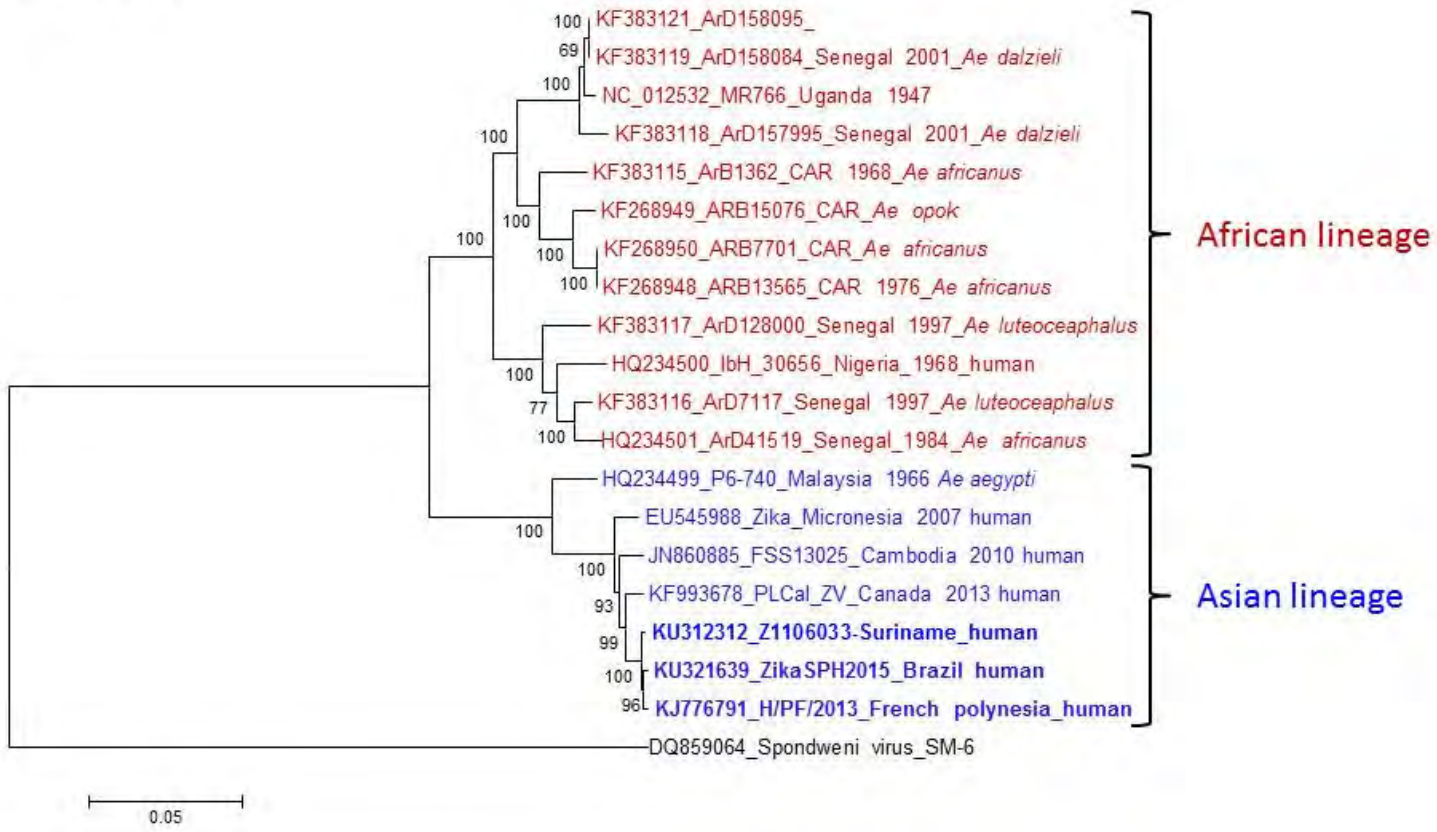


Figure 1B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KF268949_ARB15076_CAR_Ae opok
KF268950_ARB7701_CAR_Ae africanus
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HQ234499_P6-740_Malaysia 1966 Ae aegypti
EU545988_Zika_Micronesia 2007 human
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KF993678_PLCal_ZV_Canada 2013 human
KU312312_Z1106033-Suriname_human
KU321639_ZikaSPH2015_Brazil human
KJ776791_H/PF/2013_French polynesia_human
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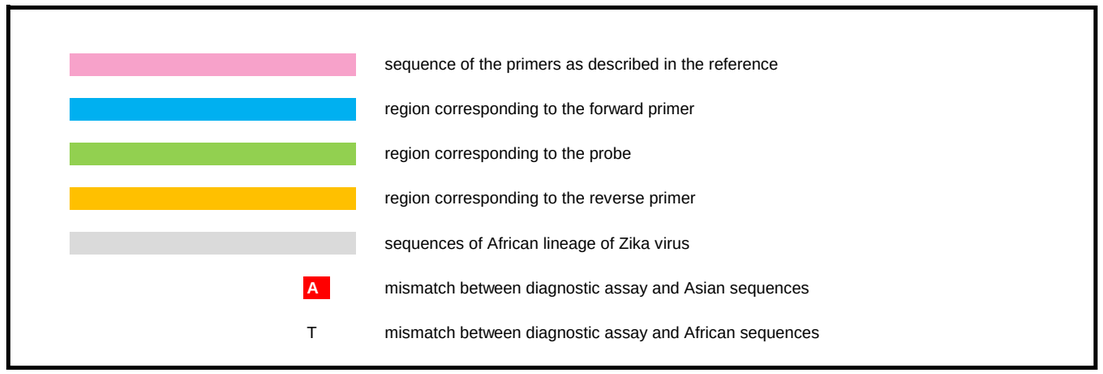
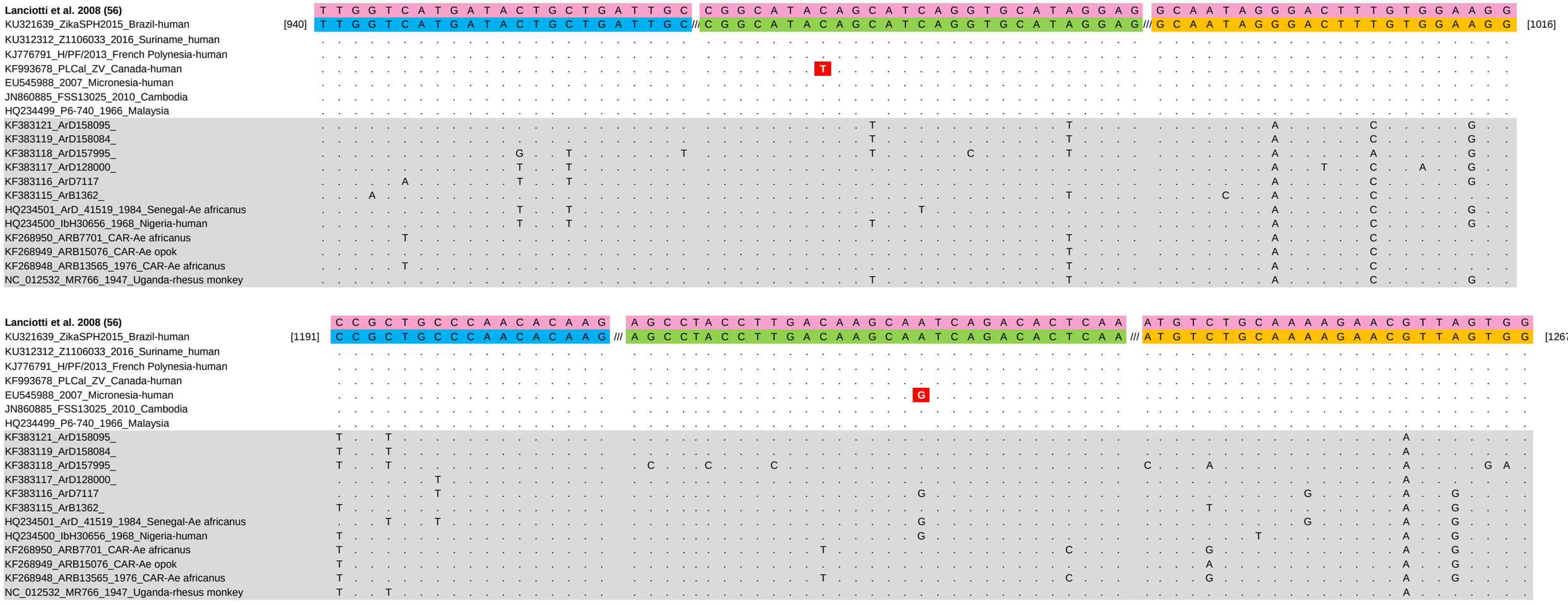
African lineage
Asian lineage
0.05

Figure 2. Overview of reverse-transcription PCR methods and their primer/probes specifics used to detect human Zika virus cases.

Figure 2.





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Tappe et al. 2014 (67)                                   T G G A G A T G A G T A T C T G T A T G       C T G A C G A A G A C C A T G C A C A C T G       C T C C T T G A C A A T A T T T A C C
KU321639_ZikaSPH2015_Brazil-human                [6012]  T G G A G A T G A G T A T C T G T A T G  ///  C T G A C G A A G A C C A T G C A C A C T G  ///  C T C C T T G A C A A T A T T T A C C  [6106]
KU312312_Z1106033_2016_Suriname_human                    . . . . . . . . . . . . . . . . . . . .       . . . . . . . . . . . . . . . . . . . . . .       . . . . . . . . . . . . . . . . . . .
KJ776791_H/PF/2013_French Polynesia-human                . . . . . . . . . . . . . . . . . . . .       . . . . . . . . . . . . . . . . . . . . . .       . . . . . . . . . . . . . . . . . . .
KF993678_PLCal_ZV_Canada-human                           . . . . . . . . . . . . . . . . . . . .       . . . . T . . . . . . . . . . . T . . . . .       . . . . . . . . . . . C . . . . . . .
EU545988_2007_Micronesia-human                           . . . . . . . . . . . . . . . . . . . .       . . . . T . . . . . . . . . . . . . . . . .       . . T . . . . . . . . C . . . . . . .
JN860885_FSS13025_2010_Cambodia                          . . . . . . . . . . . . . . . . . . . .       . . . . T . . . . . . . . . . . . . . . . .       . . T . . . . . . . . C . . . . . . .
HQ234499_P6-740_1966_Malaysia                            . . . . . . . . . . . . . . . . . . . .       . . . . . . . . . . . . . . . . . . . . . .       . . . . . . . . . . . . . . . . . . .
KF383121_ArD158095_                                      . . . . . . . . . . . . C A . . . . . .       . . . . T . . . . G . . . . . . . . . . . .       . . T . . . . . . . . C . . C . . . .
KF383119_ArD158084_                                      . . . . . . . . . . . . C A . . . . . .       . . . . T . . . . G . . . . . . . . . . . .       . . T . . . . . . . . C . . C . . . .
KF383118_ArD157995_                                      . . . . . . . . . . . . C A . . . . . .       . . . . T . . . . . . . . . . . . . . . . .       . . T . . . . . . . . C . . C . . . .
KF383117_ArD128000_                                      C . . . . . . . . . . . C A . . . . . .       . . . . T . . . . . . . . . . . . . . . . .       . . T . . . . . . . . C . . . . . . .
KF383116_ArD7117                                         . . . . . . . . . . . . C A . . . . . .       . . . . T . . . . . . . . . . . . . . . . .       . . T . . . . . . . . C . . . . . . .
KF383115_ArB1362_                                        . . . . . . . . . . . . C A . . . . . .       . . . . T . . . . . . . . C . . . . . . . .       . . T . . . . . . . . C . . C . . . .
HQ234501_ArD_41519_1984_Senegal-Ae africanus             . . . . . . . . . . . . C A . . . . . .       . . . . T . . . . . . . . . . . . . . . . .       . . T . . . . . . . . C . . . . . . .
HQ234500_IbH30656_1968_Nigeria-human                     . . . . . . . . . . . . . A . . . . . .       . . . . T . . . . . . . . . . . . . . . . .       . . T . . C . . . . . C . . . . . . .
KF268950_ARB7701_CAR-Ae africanus                        . . . . . . . . . . . . C A . . . . . .       . C . . T . . . . . . . . . . . . . . . . .       . . T . . . . . . . . C . . C . . . .
KF268949_ARB15076_CAR-Ae opok                            . . . . . . . . . . . . C A . . . . . .       . C . . T . . . . . . . . . . . . . . . . .       . . T . . . . . . . . C . . C . . . .
KF268948_ARB13565_1976_CAR-Ae africanus                  . . . . . . . . . . . . C A . . . . . .       . C . . T . . . . . . . . . . . . . . . . .       . . T . . . . . . . . C . . C . . . .
NC_012532_MR766_1947_Uganda-rhesus monkey                . . . . . . . . . . . . C A . . . . . .       . . . . T . . . . G . . . . . . . . . . . .       . . T . . . . . . . . C . . C . . . .
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Faye et al. 2013 (66)                                    A A R T A C A C A T A C C A R A A C A A A G T G G T       C T Y A G A C C A G C T G A A R       C A A G A C C A R A G R G G G A G Y G G A
KU321639_ZikaSPH2015_Brazil-human                [9376]  A A G T A C A C A T A C C A A A A C A A A G T G G T  ///  C T T A G A C C A G C T G A A A  ///  C A A G A C C A A A G G G G G A G C G G A  [9477]
KU312312_Z1106033_2016_Suriname_human                    . . . . . . . . . . . . . . . . . . . . . . . . . .       . . . . . . . . . . . . . . . .       . . . . . . . . . . . . . . . . . . . . .
KJ776791_H/PF/2013_French Polynesia-human                . . . . . . . . . . . . . . . . . . . . . . . . . .       . . . . . . . . . . . . . . . .       . . . . . . . . . . . . . . . . . . . . .
KF993678_PLCal_ZV_Canada-human                           . . . . . . . . . . . . . . . . . . . . . . . . . .       . . . . . . . . . . . . . . . .       . . . . . . . . . . . . . . . . . . . . .
EU545988_2007_Micronesia-human                           . . . . . . . . . . . . . . . . . . . . . . . . . .       . . C . . . . . . . . . . . . .       . . . . . . . . . . . . . . . . . . . . .
JN860885_FSS13025_2010_Cambodia                          . . . . . . . . . . . . . . . . . . . . . . . . . .       . . . . . . . . . . . . . . . .       . . . . . . . . . . . . . . . . . . . . .
HQ234499_P6-740_1966_Malaysia                            . . . . . . . . . . . . . . . . . . . . . . . . . .       . . . . . . . . . . . . . . . .       . . . . . . . . . . . . . . . . . . . . .
KF383121_ArD158095_                                      . . A . . . . . . . . . . . . . . . . . . . . . . .       . . C . . . . . . . . . . . . G       . . . . . . . . G . . A . . . . . T . . .
KF383119_ArD158084_                                      . . A . . . . . . . . . . . . . . . . . . . . . . .       . . C . . . . . . . . . . . . G       . . . . . . . . G . . A . . . . . T . . .
KF383118_ArD157995_                                      . . A . . . . . . . . . . . . . . . . . . . . . . .       . . C . . . . . . . . . . . . G       . . . . . . . . G . . A . . . . . T . . .
KF383117_ArD128000_                                      . . A . . . . . . . . . . . . . . . . . . . . . . .       . . C . . . . . . . . . . . . G       . . . . . . . . G . . A . . . . . T . . .
KF383116_ArD7117                                         . . A . . . . . . . . . . . . . . . . . . . . . . .       . . C . . . . . . . . . . . . G       . . . . . . . . G . . A . . . . . . . . .
KF383115_ArB1362_                                        . . A . . . . . . . . . . . . . . . . . . . . . . .       . . . . . . . . . . . . . . . G       . . . . . . . . G . . A . . . . . T . . G
HQ234501_ArD_41519_1984_Senegal-Ae africanus             . . A . . . . . . . . . . . . . . . . . . . . . . .       . . C . . . . . . . . . . . . G       . . . . . . . . G . . A . . . . . T . . .
HQ234500_IbH30656_1968_Nigeria-human                     . . A . . . . . . . . . . . . . . . . . . . . . . .       . . C . . . . . . . . . . . . G       . . . . . . . . G . . A . . . . . . . . .
KF268950_ARB7701_CAR-Ae africanus                         . . . . . . . . . - - - A . . . . . . . . . . . . .       . . C . . . . . . . . . . . . G       . . G . . . . . G . . A . . . . . T . . .
KF268949_ARB15076_CAR-Ae opok                             . . . . . . . . . . . . . . . . . . . . . . . . . .       . . C . . . . . . . . . . . . G       . . . . . . . . G . . A . . . . . T . . .
KF268948_ARB13565_1976_CAR-Ae africanus                  . . . . . . . . . - - - A . . . . . . . . . . . . .       . . C . . . . . . . . . . . . G       . . G . . . . . G . . A . . . . . T . . .
NC_012532_MR766_1947_Uganda-rhesus monkey                . . A . . . . . . . . . . . . . . . . . . . . . . .       . . C . . . . . . . . . . . . G       . . . . . . . . G . . A . . . . . T . . .
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Maher-Sturgess et al 2008 (76)                           A A Y T C I A C I C A I G A R A T G T A Y  //  T G G T W Y A T G T G G Y T I G G
KU321639_ZikaSPH2015_Brazil-human                [8307]  A A C T C T A C A C A T G A G A T G T A C      T G G T A T A T G T G G C T A G G  [9107]
KU312312_Z1106033_2016_Suriname_human                    . . . . . . . . . . . . . . . . . . . . .      . . . . . . . . . . . . . . . . .
KJ776791_H/PF/2013_French Polynesia-human                . . . . . . . . . . . . . . . . . . . . .      . . . . . . . . . . . . . . . . .
KF993678_PLCal_ZV_Canada-human                           . . . . . . . . . . . . . . . . . . . . .      . . . . . . . . . . . . . . . . .
EU545988_2007_Micronesia-human                           . . . . . . . . . . . . . . . . . . . . .      . . . . . . . . . . . . . . . . .
JN860885_FSS13025_2010_Cambodia                          . . . . . . . . . . . . . . . . . . . . .      . . . . . C . . . . . . . . . . .
HQ234499_P6-740_1966_Malaysia                            . . . . . . . . . . . . . . . . . . . . .      . . . . . C . . . . . . . . . . .
KF383121_ArD158095_                                      . . . . . C . . . . . . . . . . . . . . .      . . . . . C . . . . . . T . G . .
KF383119_ArD158084_                                      . . . . . C . . . . . . . . . . . . . . .      . . . . . C . . . . . . T . G . .
KF383118_ArD157995_                                      . . . . . C . . . . . . . . . . . . . . .      . . . . . C . . . . . . T . G . .
KF383117_ArD128000_                                      . . . . . C . . . . . . . . . . . . . . T      . . . . . C . . . . . . T . G . .
KF383116_ArD7117                                         . . . . . C . . . . . . . . . . . . . . T      . . . . . C . . . . . . T . G . .
KF383115_ArB1362_                                        . . . . . C . . . . . . . . . . . . . . T      . . . . . C . . . . . . T . G . .
HQ234501_ArD_41519_1984_Senegal-Ae africanus             . . . . . . . . . . . . . . . . . . . . T      . . . . . C . . . . . . T . G . .
HQ234500_IbH30656_1968_Nigeria-human                     . . . . . C . . . . . . . . . . . . . . T      . . . . . C . . . . . . T . G . .
KF268950_ARB7701_CAR-Ae africanus                        . . . . . C . . . . . . . . . . . . . . T      . . . . . C . . . . . . T . G . .
KF268949_ARB15076_CAR-Ae opok                             . . . . . C . . . . . . . . A . . . . . T      . . . . . C . . . . . . T . G . .
KF268948_ARB13565_1976_CAR-Ae africanus                  . . . . . C . . . . . . . . . . . . . . T      . . . . . C . . . . . . T . G . .
NC_012532_MR766_1947_Uganda-rhesus monkey                . . . . . C . . . . . . . . . . . . . . .      . . . . . C . . . . . . T . G . .
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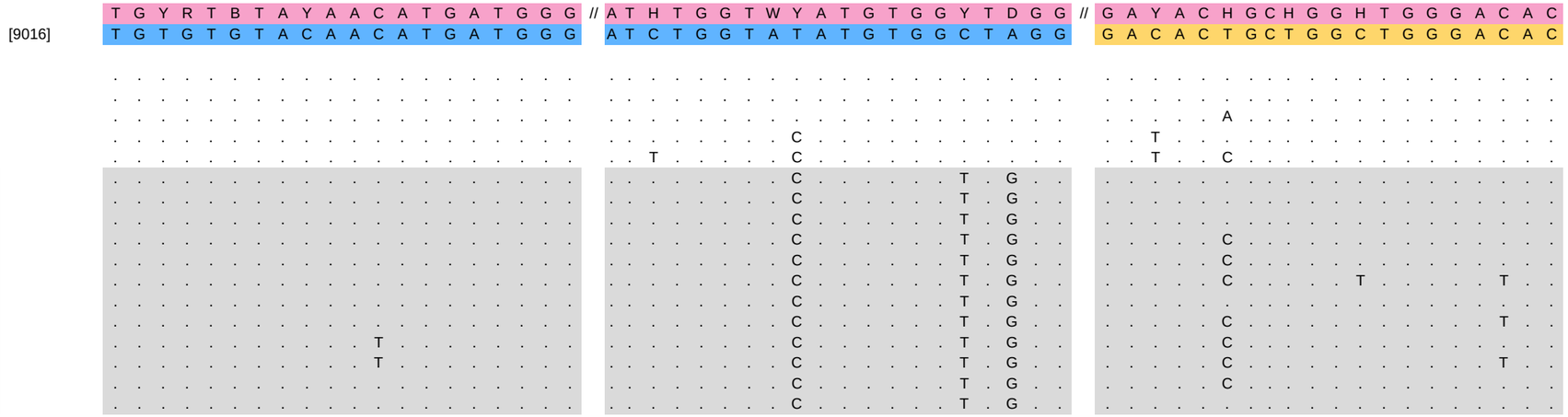
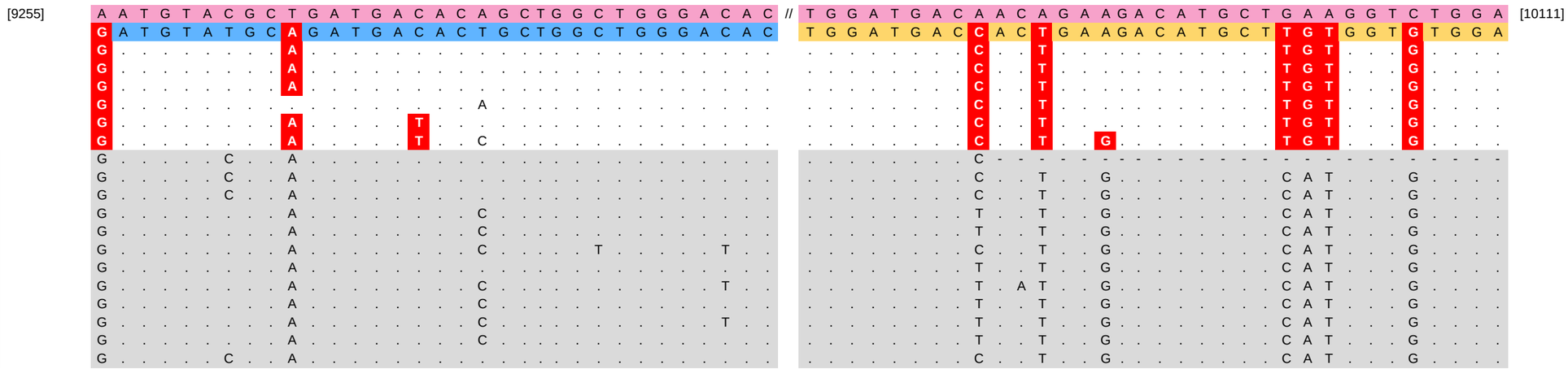
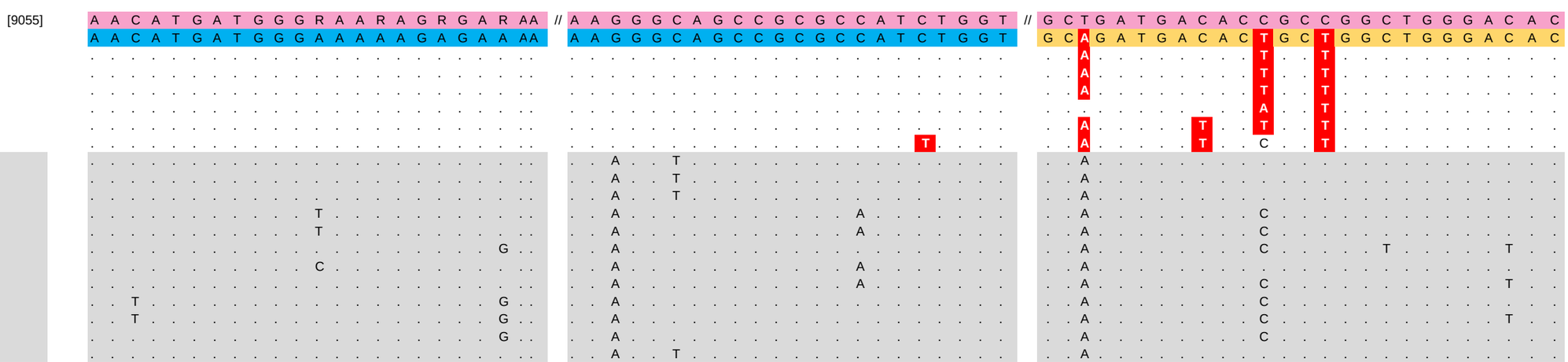
Moureau et al 2007 (72)



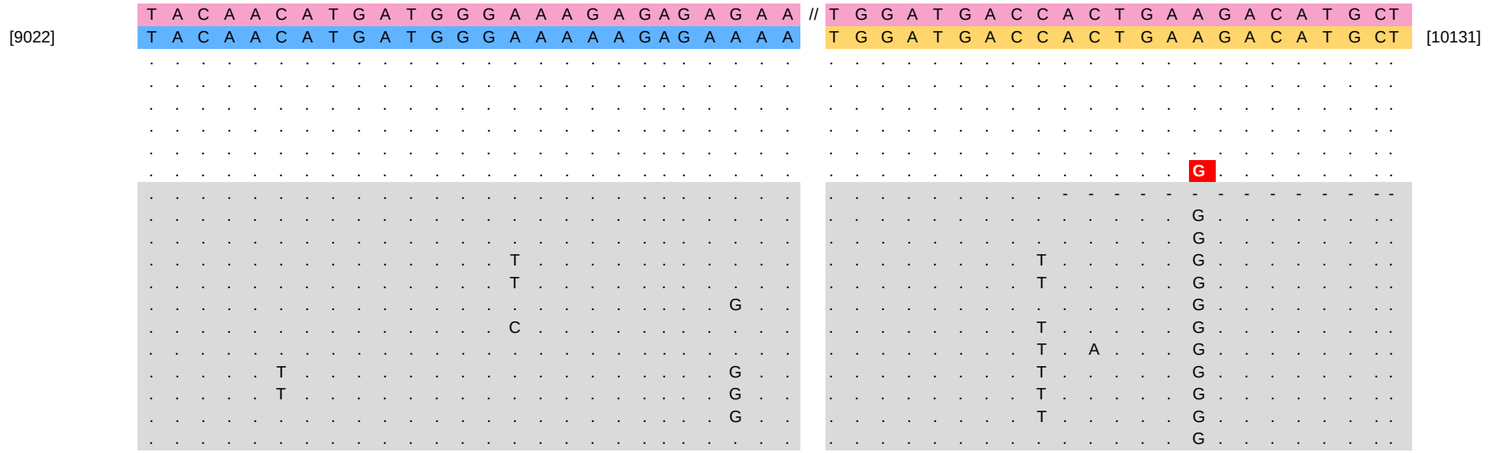
Ayers et al 2006 (75)



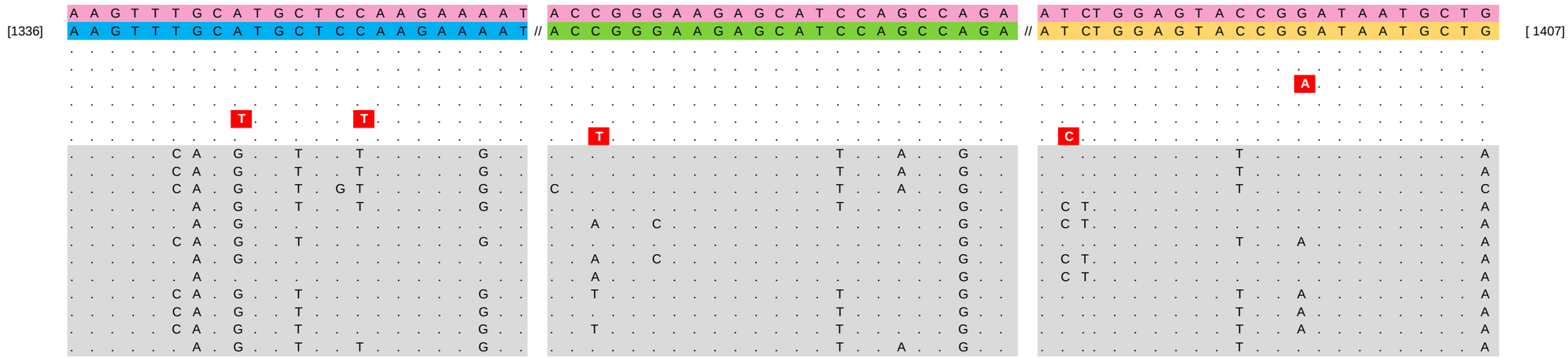
Scaramozzino et al 2001 (74)



Kuno et al 1998 (73) / FU1-cFD3



Pyke et al 2014 (81) envelope



Pyke et al 2014 (81) / NS1