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Challenges in diagnosis and management of chikungunya and Zika virus infections

Ralph Huits

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for the degree of Doctor in Biomedical Sciences

Promotor:
prof. dr. Jan A. Jacobs
Co-promotors:
prof. dr. Emmanuel Bottieau
Dr. Marjan Van Esbroeck
dr. Lieselotte Cnops

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Composition of the Examination Committee

Promotor:

Prof. dr. Jan Jacobs	KU Leuven
	Institute of Tropical Medicine Antwerp

Co-promotors:

Prof. dr. Emmanuel Bottieau	Institute of Tropical Medicine Antwerp
Dr. Marjan Van Esbroeck	Institute of Tropical Medicine Antwerp
dr. Lieselotte Cnops	Institute of Tropical Medicine Antwerp

Jury:

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dr. Dirk Jochmans	KU Leuven
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Prof. dr. David Hamer	Boston University School of Public Health

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Blind Men and the Elephant

A Poem by John Godfrey Saxe (1816-1887)

It was six men of Indostan,
To learning much inclined,
Who went to see the Elephant
(Though all of them were blind),
That each by observation
Might satisfy his mind.

The First approach'd the Elephant,
And happening to fall
Against his broad and sturdy side,
At once began to bawl:
"God bless me! but the Elephant
Is very like a wall!"

The Second, feeling of the tusk,
Cried, -"Ho! what have we here
So very round and smooth and sharp?
To me 'tis mighty clear,
This wonder of an Elephant
Is very like a spear!"

The Third approach'd the animal,
And happening to take
The squirming trunk within his hands,
Thus boldly up and spake:
"I see," -quoth he- "the Elephant
Is very like a snake!"

The *Fourth* reached out an eager hand,
And felt about the knee:
"What most this wondrous beast is like
Is mighty plain," -quoth he,-
"'Tis clear enough the Elephant
Is very like a tree!"

The *Fifth*, who chanced to touch the ear,
Said- "E'en the blindest man
Can tell what this resembles most;
Deny the fact who can,
This marvel of an Elephant
Is very like a fan!"

The *Sixth* no sooner had begun
About the beast to grope,
Then, seizing on the swinging tail
That fell within his scope,
"I see," -quoth he,- "the Elephant
Is very like a rope!"

And so these men of Indostan
Disputed loud and long,
Each in his own opinion
Exceeding stiff and strong,
Though each was partly in the right,
And all were in the wrong!

MORAL,
So, oft in theologic wars
The disputants, I ween,
Rail on in utter ignorance
Of what each other mean;

And prate about an Elephant
Not one of them has seen!

Introduction

As a clinician practicing in endemic areas and at the travel clinic of the Institute of Tropical Medicine in Antwerp, the study of emerging arbovirus infections has made me feel like a blind man describing an elephant on more than one occasion. I have been fortunate to meet and work with many fellow 'blind men' across a range of clinical and scientific disciplines: clinical biologists, virologists, molecular biologists, laboratory technicians, entomologists, epidemiologists, infectious disease modelers, public health officials and policy makers … . They all helped me to comprehend the stories of individual patients who consulted me, and they continue to improve my skills in diagnosing and managing arbovirus infections.

Arthropod-borne viruses (arboviruses) are viruses that are transmitted by arthropods, e.g. mosquitoes, ticks or sandflies. During my PhD studies, the epidemics of chikungunya virus (CHIKV) and Zika virus (ZIKV) in the Americas made front page news. These two arboviruses belong to different virus families, *Togaviridae* and *Flaviviridae*, respectively. They are transmitted by the same vector species, the widely distributed *Aedes* mosquitoes. The ever expanding suitable habitats for these vectors (notably *Aedes aegypti* and *Aedes albopictus*) have facilitated the rapid emergence and global spread of CHIKV, ZIKV and other arboviruses. Over the past decades, millions of people in Africa, Asia, Europe and the Americas were infected with CHIKV or ZIKV. Today more than 3 billion people (approximately half of the world population) are at risk of acquiring these infections (1–4).

CHIKV and ZIKV also share the ability to harm public health and individual well-being beyond the acute stages of infection. The large scale of the CHIKV and ZIKV outbreaks has revealed unexpected and sometimes severe complications (1). CHIKV infection often results in long-term arthralgia or arthritis, that impairs quality of life (5,6). ZIKV infection in humans was previously considered to be asymptomatic, or to result in mild illness only. During the 2015 outbreak in Brazil, ZIKV infection was associated with poor neonatal outcomes in women who were infected during pregnancy, and an association of ZIKV infection with neurological manifestations was found. Also, evidence emerged

that in addition to vector-borne distribution, ZIKV could be transmitted via sexual intercourse (7). Physicians working in internal medicine, family medicine, neurology, obstetrics, paediatrics, rheumatology and radiology, may encounter patients with CHIKV or ZIKV infections, or with complications of these infections.

As is clarified in the paragraphs below, the diagnosis of arbovirus infections is not straightforward. Certainly, pattern recognition of some clinical syndromes is helpful to include medically important arbovirus infections in the differential diagnosis. Common patterns caused by arbovirus infections are acute febrile disease, arthralgia with rash, haemorrhagic symptoms, or neurological syndromes (Table 1.) (8). Knowledge of the geographic distribution and epidemic circulation of arboviruses can increase the index of suspicion. Obtaining a detailed history and a timeline of signs and symptoms that a person experienced since disease onset, and relating this sequence to the known intrinsic incubation period (i.e. the time between infection and onset of the disease or infectiousness) of a suspected arbovirus infection is an elementary step in assessing the odds. However, in the consultation room or at bedside, symptoms of CHIKV, ZIKV and other arbovirus infections frequently resemble those of illness with other etiology (9).

Therefore, a definitive diagnosis can only be ascertained by laboratory evaluation. The choice of appropriate diagnostic test methods and the interpretation of the test results depends on the timing of assessment in relation to the course of infection. The frequent occurrence of false positive results, particularly when indirect methods such as antibody detection assays are employed, warrants a careful evaluation of previous exposure to arbovirus infection or vaccination (8,10).

In this thesis I examined risk factors, clinical presentation and diagnostic challenges of CHIKV and ZIKV infections and their implications for the management of these arbovirus infections. In the introduction, I presented a background on the formidable threat of *Aedes*-borne infections to human health worldwide, to key concepts in arbovirology, and to CHIKV and ZIKV infection in particular.

Arthropod-borne viruses

Arthropod-borne viruses (arboviruses) are defined by the World Health Organization as "Viruses that are maintained in nature principally, or to an important extent, through biological transmission between susceptible vertebrate hosts by hematophagous arthropods or through trans-ovarian and possibly venereal transmission in arthropods" (11). More than 500 species of arbovirus in 14 different virus families have been identified (12,13). The definition by mode of transmission captures the complex interaction between virus, arthropod vectors and vertebrates. Biological transmission (as opposed to mechanical or accidental transmission) refers to the cycle of viral replication in the arthropod and transfer of virus from the arthropod to the vertebrate host during a bloodmeal. The maintenance of this transmission cycle requires that the replication of virus in the vertebrate host produces sufficient viremia (i.e. presence of virus in the blood) to pass the virus on to a next suitable vector that feeds on the host (14). More than one hundred arboviruses are known to cause human or animal infections (12). These viruses can be classified in 5 different families. A selected overview of medically important arboviruses is presented in Table 1 (adapted from Cleton et al. (8)). A large proportion of these medically important arboviruses, including CHIKV and ZIKV, are transmitted by mosquitoes.

Infection of humans by mosquito-borne arboviruses can occur as a result of enzootic, epizootic or urban epidemic transmission cycles (Fig. 1) (15). The mechanism of the enzootic and epizootic (or rural) cycle is spillover infection, where the vector accidentally feeds on a human host and transmits viruses that are typically maintained in wildlife or domesticated animals, respectively. The level of viremia resulting from these infections is often too low to sustain mosquito-borne transmission between humans.

Table 1. medically important arboviruses

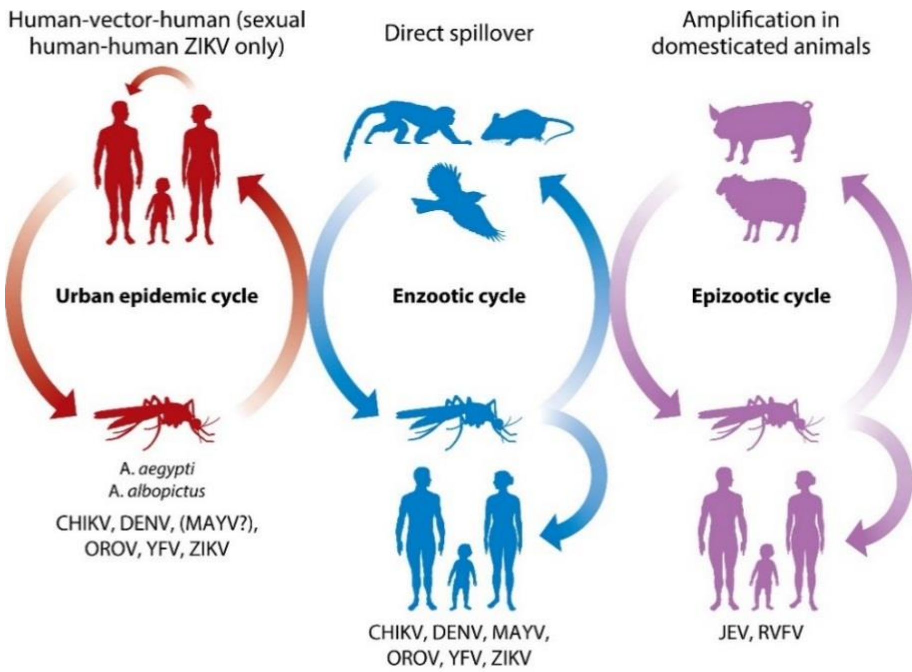
Family / Genus	Virus	Vector	Host	Transmission cycle	Incubation period	Clinical syndrome
Nairoviridae						
Orthonairovirus	Crimean-Congo hemorrhagic fever	Tick	Birds, small mammals	R; H2H	1–3 (1–9)	FD, HS, (NS)
Peribunyaviridae						
Orthobunyavirus	Bwamba virus	Mosquito	Unknown	R	1–14	FD, AR, (NS)
	Bunyamwera virus	Mosquito	? rodents	R	Unknown	FD, AR, (NS)
	Guaroa virus	Mosquito	Unknown	R	Unknown	FD, AR
	Ilesha virus	Mosquito	Unknown	R (U)	Unknown	FD, AR, (NS, HS)
	Ngari virus	Mosquito	Unknown	R	Unknown	FD, AR, HS
	La Crosse virus	Mosquito	Small mammals	R	5–15	FD, NS
	Tahyna virus	Mosquito	Rodents, small mammals	U	3–7	FD, AR, (NS), conjunctivitis, pneumonia
	Oropouche virus	Midge	Humans, Sloths, ? primates/birds	R, U	4–8	FD, AR, NS
	Tataguine virus	Mosquito	Unknown	R	Unknown	FD, AR
Phlebovirus	Toscana virus	Sandfly	Humans, bats	R	2–14	FD, NS, (AR)
	Sandfly fever Naples/Sicilian	Sandfly	Humans, rodents	R	2–14	FD
	Rift valley fever virus	Mosquito	Rodents, bats, cattle	R; H2H	1–7	FD, HS, NS, hepatitis
Flaviviridae						
Flavivirus	Dengue virus	Mosquito	Primates, humans	R, U; H2H	4–7 (3–14)	FD, HS, AR
	Japanese encephalitis virus	Mosquito	Ardeid birds, pigs	R, U	5–14	FD, NS
	West Nile virus	Mosquito	Birds	R, U; H2H	3–5 (2–14)	FD, NS, (AR)
	St. Louis encephalitis virus	Mosquito	Birds	R, U	2–21	FD, NS
	Murray Valley virus	Mosquito	Ardeid birds	R	1–28	FD, NS
	Kyasanur Forest disease virus	Tick	Small mammals, humans	R	3–8	FD, HS, conjunctivitis, pneumonia

Flavivirus (continued)	Alkhurma hemorrhagic fever virus	Tick	Small mammals	R	3–12	FD, HS
	Tick-borne encephalitis virus	Tick	Small mammals, birds	R; H2H	7–14	FD, NS, (HS)
	Ilheus virus	Mosquito	Birds	R	Unknown	FD, NS
	Yellow fever virus	Mosquito	Primates, humans	R, U; H2H	3–6	FD, HS, hepatitis
	Zika virus	**Mosquito**	**Primates, humans**	**R, U; H2H**	**3-12**	**FD, AR, NS, conjunctivitis, congenital syndrome**
Reoviridea						
Coltivirus	Colorado tick fever virus	Tick	Small mammals	R; H2H	3–5 (0–20)	FD, NS, AR, HS
Seadronvirus	Banna virus	Mosquito	Unknown	R	Unknown	FD, AR, NS
Togaviridae						
Alphaviruses	Barmah Forest virus	Mosquito	Birds, marsupials	R, U	7–9 (5–2)	FD, AR
	Eastern equine encephalitis virus	Mosquito	Birds, small mammals, marsupials	R	3–10	FD, NS
	Chikungunya virus	**Mosquito**	**Primates, humans**	**R, U**	**3–7** (1–12)	**FD, AR, (HS), (NS), Conjunctivitis**
	Mayaro virus	Mosquito	Primates	R, U	6–12 (3–12)	FD, AR, HS
	O'Nyong Nyong virus	Mosquito	Primates, humans	R, U	>8	FD, AR
	Ross river virus	Mosquito	Marsupials, mammals	R, U	7–9 (3–21)	FD, AR, HS
	Sindbis virus	Mosquito	Birds	R	1–7	FD, AR
	Western equine encephalitis virus	Mosquito	Birds, small mammals	R	2–10	FD, NS
	Venezuelan equine encephalitis virus	Mosquito	Small mammals	R	<1–5	FD, NS

Transmission cycle: *R = rural (enzootic and epizootic), U = urban; H2H = human to human transmission reported*
Clinical syndromes: *FD = febrile disease; AR = arthralgia/ rash; HS = haemorrhagic syndrome; NS = neurological syndrome; () = less frequent.*

When virus replication in the human host results in infection of feeding anthropophilic mosquitoes, an enzootic cycle can evolve into a 'human-mosquito-human', or urban epidemic cycle. CHIKV, ZIKV, dengue virus (DENV) and yellow fever virus (YFV) have successfully established an urban epidemic cycle of transmission in the presence of efficient mosquito vectors, such as *Aedes aegypti* and *Aedes albopictus* (15).

Figure 1. Transmission cycles of mosquito-borne arboviruses



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Aedes aegypti and *Aedes albopictus*

Female mosquitoes use proteins and iron from the blood of vertebrates for egg production. The mosquito can become infected during a bloodmeal from a viremic host with virus and, after an extrinsic incubation period (i.e. the time between infection by feeding and presence of virus in the vectors' saliva) transmit the virus to another host. Mosquitoes that belong to the genus *Aedes*, subgenus *Stegomyia*, are capable vectors of viruses that cause human disease (14). The extent to which arthropods are able to transmit pathogens is referred to as vector capacity. Vector capacity is determined by the arthropods' susceptibility to infection, the feeding habits and the lifespan of the vector. The identification of *Aedes* mosquitoes as vectors of arboviruses such as YFV, DENV, ZIKV and CHIKV requires fulfilment of four criteria (16):

(1) Isolation of the virus from mosquitoes collected in the field.
(2) Demonstration of experimental infection of the mosquito following a virus-containing meal.
(3) Demonstration of the presence of virus in salivary fluids, or demonstration of transmission of virus by biting a vertebrate host.
(4) Confirmation from field studies that the mosquito species is associated with the vertebrate population in which the virus infection is occurring.

Aedes aegypti is highly susceptible to infection with several arboviruses (*e.g.* YFV, DENV) and it is the principal vector of CHIKV and ZIKV. This species is mainly found in tropical or sub-tropical climates. Following effective eradication campaigns in the 1940s and 50s, *Ae. aegypti* was eradicated in large parts of Latin America and the Caribbean. However, it quickly re-invaded these territories from areas that failed to eradicate the vector after cessation of the campaigns in the 1970s (17). *Ae. aegypti* are anthropophilic day-time biters, and mosquitoes feeding on human blood were found to have a reproductive advantage over those feeding

on sugar (18). *Ae. aegypti* mosquitoes often feed on multiple human hosts per day, thereby increasing the likelihood being infected with, and transmitting arboviruses (19,20). Because its flight range is usually limited to 100 meters, urban environments with dense population constitute a suitable habitat for this mosquito. Peridomestic water collections form ideal sites for laying eggs (oviposition), that in a dry state can survive colder temperatures for longer than one year. When temperatures rise and when the eggs are immersed in water, they hatch and develop into larval and pupal stages in approximately one week. Then, the adult mosquitoes emerge from their underwater pupae (Fig. 2).

The extrinsic incubation period for CHIKV is very short. Viral dissemination and transmission of Asian genotype CHIKV in the Caribbean by *Ae. aegypti* can occur as soon as 2 days, and peaks at 5 days after ingesting an infective bloodmeal (21,22).

The susceptibility of *Ae. aegypti* to infection with ZIKV strains that were isolated during the outbreak in Brazil was higher than mosquito susceptibility to ZIKV strains from Africa and Asia. The extrinsic incubation time was reduced (to 3 days) (23). A single nucleotide substitution in the NS1-coding region of the Brazilian ZIKV isolates resulted in an alanine-to-valine amino acid substitution at residue 188 of the NS1-protein and facilitated acquisition of ZIKV by *Ae. aegypti* (24). This evolutionary enhancement of ZIKV infectivity led to increased vector capacity of *Ae. aegypti* for American ZIKV strains and could have contributed to the magnitude of the ZIKV outbreak in the Americas (24).

Aedes albopictus is often referred to as the Asian tiger mosquito because of its geographic origin and the distinctive white markings on body and legs (Fig. 4). It used to be a forest-dwelling species, but it has adapted rapidly to temperate climates and urban environments, where it breeds in artificial water containers (25). The eggs of *Ae. albopictus* can enter a dormant state when the temperature drops below 10°C. International travel and trade facilitate dispersal of eggs (26). Under permissive environmental conditions, the eggs hatch and *Ae. albopictus* larvae successfully compete for resources with other species (e.g. *Ae. aegypti*), leading to reduction or displacement of resident mosquito populations

(26). These characteristics and ecological adaptability made the Asian tiger mosquito rank among the most invasive insect species (27). *Ae. albopictus* is also a daytime biter. While preferring human blood, it also feeds on animals. This 'zoophilic' feeding behavior increases the exposure to pathogens, and the ability to transmit them. *Ae. albopictus* has been shown to be capable of transmitting many viruses, including CHIKV and ZIKV (25,28,29).

Although *Ae. albopictus* was historically considered a less competent vector than *Ae. aegypti*, it was confirmed as the main vector species in the 2005-2007 CHIKV outbreak in the Indian Ocean region, and as the only vector in the first CHIKV outbreaks in Europe (26,30,31). CHIKV strains from these outbreaks carried an alanine to valine substitution at position 226 in the E1 envelope glycoprotein (E1-A226V), that resulted in significantly enhanced infectivity for *Ae. albopictus* (30,32,33). This is a prime example of the dynamic nature of the interaction between virus and vector.

Only one study (from Gabon) reported *Ae. albopictus* as the primary vector of a ZIKV outbreak (28). A study from Singapore showed hundred percent of *Ae. albopictus* mosquitoes to be infectious, ten days after ingestion of a ZIKV strain from Uganda (MR766) (29). *Aedes* species susceptibility seems to vary between mosquito strains (34). Nevertheless, even at low vector competence, the convergence of dense mosquito populations with large numbers of vertebrate hosts who are susceptible to a specific arbovirus may drive large-scale epidemics, such as the CHIKV and ZIKV outbreaks in the Americas.

The global emergence of CHIKV and ZIKV infections is closely associated with the increasing distribution of *Aedes* species. The factors that facilitate the spread of these vectors and the expansion of territories that are suitable habitats are often attributed to human activities in a modern world, such as increasing international travel and trade, urbanization and climate change (1,14). Surveillance of the expanding vector habitats is important to assess the potential for future arbovirus outbreaks. In Europe, the presence of *Ae. albopictus* continues to increase (Fig. 5).

Figure 2. ***Aedes aegypti*** **mosquito, emerging from its underwater pupa**

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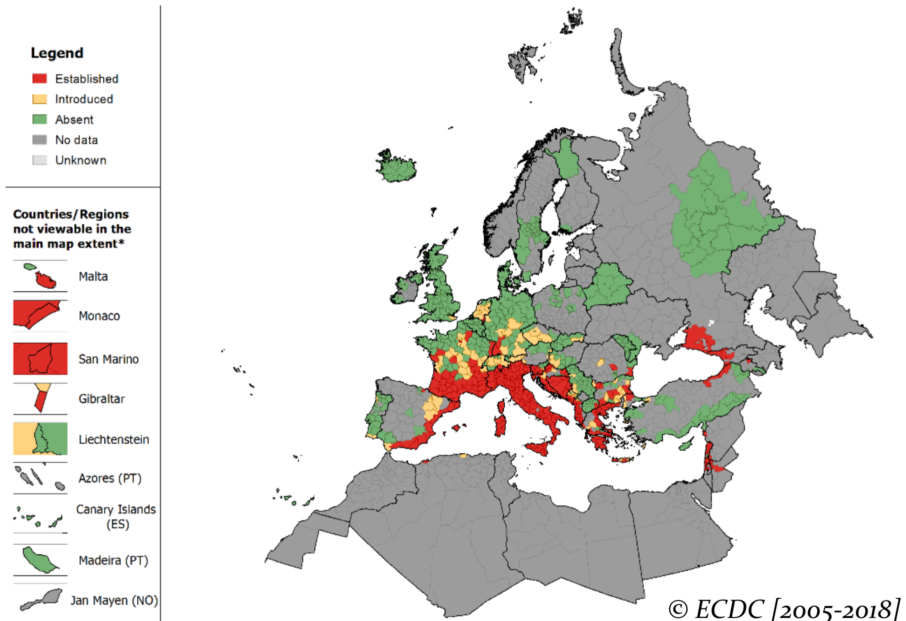
Figure 3. **Adult female *Aedes aegypti* on human skin**

Adult female Aedes aegypti can be recognized by the 'lyre' shaped marking on their dorsal thorax. Photograph by James Gathany (CDC), Public Domain.

Figure 4. Adult female *Aedes albopictus* on human skin

Adult Aedes albopictus mosquitoes have distinctive white markings on their black body. Photograph by Shutterstock ©.

Figure 5. Distribution of *Ae. albopictus* in Europe, June 2018



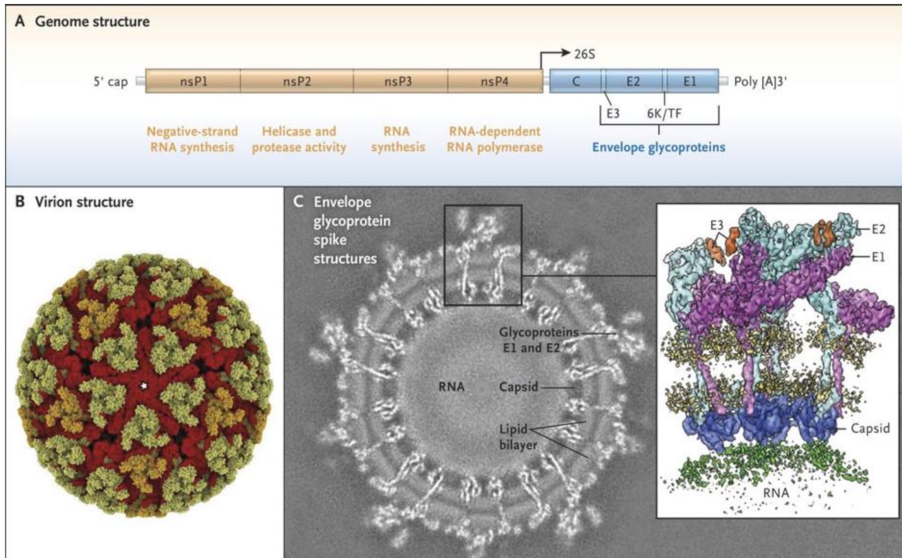
Chikungunya virus

Virology

Chikungunya virus belongs to the genus *Alphavirus*, family *Togaviridae*, that also includes o'nyong'nyong virus, Sindbis virus, Ross River virus, Venezuelan equine encephalitis virus and Mayaro virus. The viral particle is an icosahedral capsid surrounded by a lipid envelope, that measures approximately 60-70 nm in diameter. Alphaviruses have a single-stranded, positive-sense RNA genome that encodes four non-structural proteins (nsP1-4) and three structural proteins (capsid (C), envelope glycoprotein 1 (E1), envelope glycoprotein 2 (E2)) and three peptides (6K, transframe (TF), and E3) (Fig. 6A). The structural proteins are translated from subgenomic RNA as a polyprotein, and processed into mature capsid protein and envelope proteins E1 and E2. The capsid protein C then forms nucleocapsids by associating with genomic RNA copies. Upon release from the host cell, the new virus particles are coated with the envelope glycoproteins E1 and E2. E1-E2 heterodimers form trimeric spikes on the viral surface (Fig. 6B and 6C). E2 mediates entry into a new host cell, after binding to unidentified receptors. E1 is exposed at low pH and initiates fusion and release of the nucleocapsid (35–37). Because the non-structural proteins are not assembled into the new virions, the envelope glycoproteins of CHIKV are the primary antigenic targets for the host immune response (38).

CHIKV was first isolated from a febrile patient in Tanzania in 1952 (39). Since then, three genotypes of the CHIKV have been identified, each with distinct antigenic characteristics (40,41). The genotypes were named after the geographical region where they were first isolated: East/Central/South African (ECSA), West African (WA) and Asian genotype.

Figure 6. Chikungunya virus genetic and physical structure.



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Epidemiology

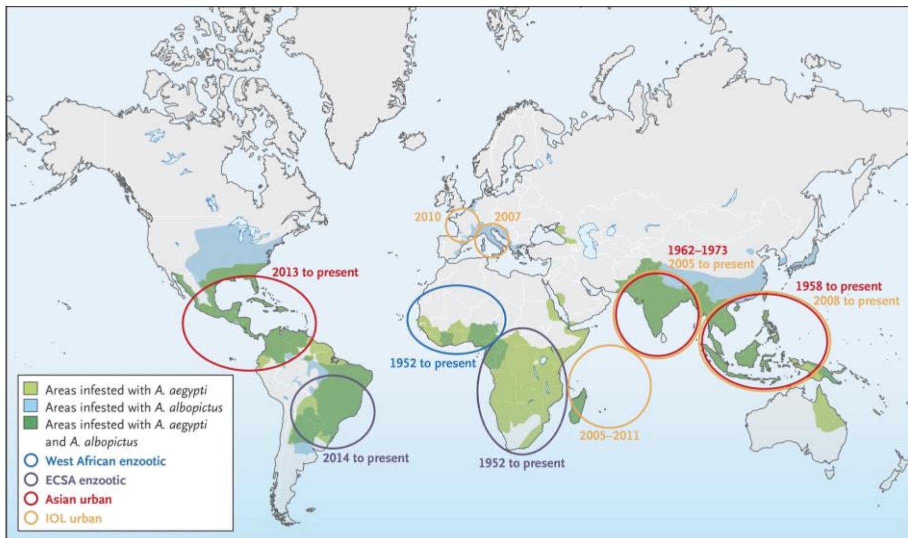
Prior to 2004

After the isolation and description of CHIKV in 1952 and throughout the second half of the twentieth century, multiple small-scale epidemics have been reported from African countries (Zimbabwe, Democratic Republic of Congo, Zambia, Senegal, Uganda, Nigeria, Angola and the Central African Republic). Sporadic human cases were reported from Cameroon and Cote d'Ivoire (41). It is likely that these outbreaks were caused by spillover infection from enzootic transmission cycles. In 1999, an outbreak involving 50,000 persons was documented in Kinshasa, Democratic Republic of the Congo. CHIKV isolates from this urban outbreak clustered in the Central African lineage (42). In Southeast Asia (Thailand, Cambodia, Myanmar, Vietnam and Indonesia) and the Indian subcontinent, outbreaks of Asian genotype CHIKV involving thousands of persons occurred frequently from 1958 onwards (41). The Asian epidemics followed urban transmission cycles with the ubiquitous *Ae. aegypti* as the main vector.

After 2004

After a massive CHIKV outbreak on Lamu island, Kenya in 2004, which involved 13,500 cases (70% of the island's population), the virus spread to Mombasa and the Comoros (43). By 2005 it had reached other islands in the Indian Ocean (La Réunion, Mauritius, and Seychelles). With over 200,000 cases infected, attack rates in La Réunion were as high as 35% of the population (44,45). The vector implicated in transmission of the ECSA CHIKV strain at La Réunion, but not in Kenya or the Comoros was *Ae. albopictus*. It was demonstrated by genetic analysis that an A226V mutation had occurred in the CHIKV envelope glycoprotein E1, which greatly enhanced replication of the virus in this mosquito species (32,33,46). The strain of ECSA genotype CHIKV that carries the A226V mutation is commonly referred to as the Indian Ocean Lineage (IOL). The combination of efficient transmission by *Ae. albopictus* and dense, immunologically naive human populations led to further explosive outbreaks across Asia and the Pacific region (Fig. 7) (41).

Figure 7. Origin, spread, and distribution of chikungunya virus and its vectors.



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CHIKV, once considered a 'tropical' infection, has also caused autochthonous outbreaks in European countries where *Ae. albopictus* is present. In Italy, clusters of transmission of IOL-lineage CHIKV were reported in 2007 and 2017 (30,47,48). In France, a local outbreak was reported in 2014 (49).

CHIKV emergence in the Americas

Circulation of CHIKV in the New World was reported for the first time on the island of Saint Martin in the Caribbean in December 2013 (50). Given the rapid geographic expansion of the ECSA-IOL lineage in Asia, the strains from the Caribbean unexpectedly clustered in the Asian genotype. They were phylogenetically related to lineages from Indonesia, China and the Philippines (21). The widespread distribution of *Ae. aegypti*, a susceptible population and the intensity of travel and trade in the region facilitated rapid spread of CHIKV across the continent. From 2013 to 2015, more than a million cases were reported from the Caribbean, Mexico and Central America, United States and South America, including Brazil and Andean countries (51).

In addition to circulation of the Asian genotype CHIKV, an ECSA genotype that did not have the A226V substitution was detected in Brazil in 2014 (52). The presumed index case was a returning traveller who was infected in Angola.

Clinical presentation of CHIKV infection

CHIKV infection results in an acute febrile disease that lasts 10-14 days in the majority of cases (53). Studies in blood donors from La Réunion suggest that asymptomatic seroconversion occurs in less than 15% of cases (54). Arthralgia is the hallmark of infection by CHIKV, that is often labeled arthritogenic along with other alphaviruses. In 1952, the severity and sudden onset of the joint pains was called chikungunya by the Makonde people of Tanzania: "that which bends up" (55). The joint pains are usually symmetric and affect peripheral and large joints in the arms and legs, but all joints may be involved. Myalgia, headache and rash are frequently observed. CHIKV infection can present with

gastrointestinal symptoms, such as diarrhea and abdominal pain. Hemorrhage as seen in dengue infections is rare in chikungunya disease. Neurological manifestations of chikungunya infection such as encephalitis are rare in the absence of comorbidities, but do occur in newborns or in patients with pre-existing liver disease. Neonates and infants, as well as elderly people have a higher risk of complications of acute CHIKV infection than healthy adults do (35). Although mortality from CHIKV infection is thought to be rare, increases in overall mortality following CHIKV outbreaks have been reported (56,57). The burden of CHIKV morbidity is aggravated by joint pains that can last for years after the acute phase and may resemble rheumatoid arthritis. Post-chikungunya chronic polyarthralgia (pCHIK-CPA) affects up to 60% of patients infected with CHIKV (53).

Currently, there are no specific antiviral interventions to treat, and no licensed vaccines to prevent CHIKV infection. Supportive treatment with analgesics and anti-inflammatory medication are used to mitigate joint pains. The role of ‘disease modifying anti-rheumatic drugs’ (DMARDs) in the management of chronic CHIKV disease is under investigation (58).

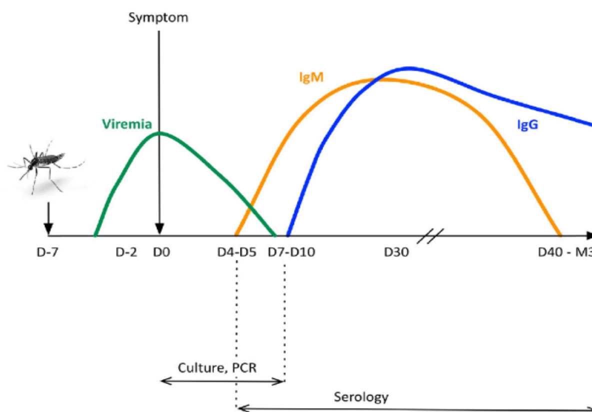
Diagnosis

Laboratory diagnosis of arbovirus infections relies on detection of the virus, viral structural components such as antigens or nucleic acid (direct methods), or the host immunologic response to the virus (indirect methods). The selection of appropriate tests for confirmation of infection is therefore not only determined by the analytical sensitivity and specificity of various assays, but it also depends on the kinetics of viremia and antibody response to infection (Fig. 8). In CHIKV infection, high viral loads (up to 10^9 copies per mL) are typically present in a patient’s serum during the first week after symptom onset (59). In this timeframe, culture of CHIKV in mammalian or insect cell lines permits a definitive diagnosis and further characterization of the isolated strains (35,53). Isolation of CHIKV, a biosafety level 3 pathogen, has no place in routine diagnostics because of slow turn-around times, technological requirements and expertise, and economic cost. Detection of amplified

CHIKV RNA by reverse transcription polymerase chain reaction (RT-PCR) or related methods has a high sensitivity within 5-7 days of symptom onset (60–63). Genetic differences between CHIKV strains may affect the sensitivity of RT-PCR for viral RNA detection, and primers and probes should be selected that detect African and Asian genotypes (61).

Many CHIKV-infected patients seek consultation only after the early phase, because of persisting joint pains. After the first week, detection of CHIKV-specific antibodies in serum by indirect immunofluorescence (IFA) or Enzyme Linked ImmunoSorbent Assay (ELISA) is the preferred diagnostic method. The antibody detection assays for the diagnosis of CHIKV are sensitive for all three genotypes, due to extensive serological cross-reactivity between the lineages (and other alphaviruses) (40,64). An immunoglobulin (Ig)M response develops around 4 to 5 days after symptom onset, and is considered diagnostic of infection in a single sample. IgM antibodies to CHIKV persist for several months, but may be found longer, possibly due to persistence of viral antigens in association with chronic disease (65). By the end of the first week IgG is usually detectable in serum (60,61,66,67). When IgM is not present simultaneously, follow-up sampling to demonstrate a fourfold increase in IgG titer is required for diagnosis.

Figure 8. Contribution of RT-PCR and antibody detection assays to diagnosing chikungunya virus in function of time after infection.



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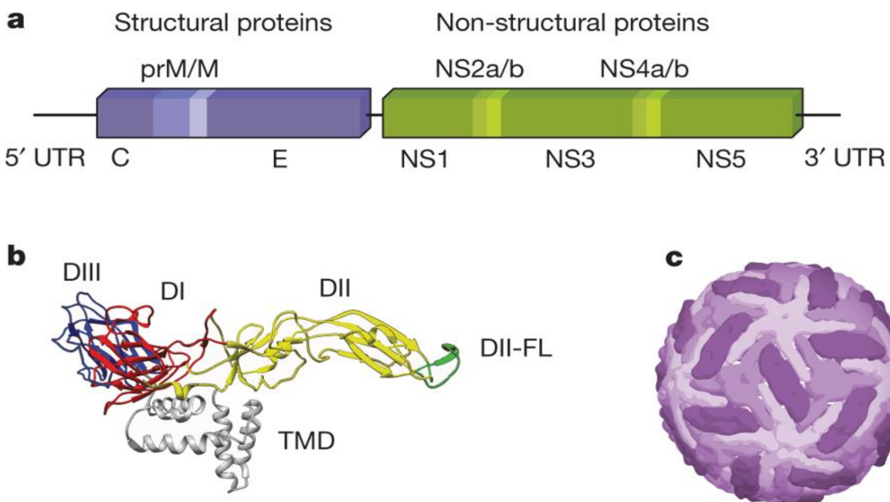
Zika virus

Virology

ZIKV belongs to the genus *Flavivirus*, family *Flaviviridae*. The members of this genus (eg. yellow fever virus, dengue virus, West Nile virus, tickborne encephalitis virus and Japanese encephalitis virus) encapsulate a positive-sense RNA genome that encodes a single polyprotein in a single open reading frame. After translation it is cleaved into three structural proteins (capsid, membrane, and envelope) and seven non-structural proteins, designated NS 1, 2a, 2b, 3, 4a, 4b and 5 (Fig 9a). The envelope protein E consists of three ectodomains (E-DI (red), E-DII (yellow) and E-DIII (blue), and is anchored into the viral membrane by two anti-parallel transmembrane domains (TMD) (grey). A highly conserved fusion loop (DII-FL) is located at the distal end of DII (green) (Fig 9b.). Mature virions have an icosahedral symmetry (Fig. 9c).

There are two geographically distinct lineages of ZIKV, an African and an Asian genotype (69,70).

Figure 9. Genomic organization and structure of Zika virus



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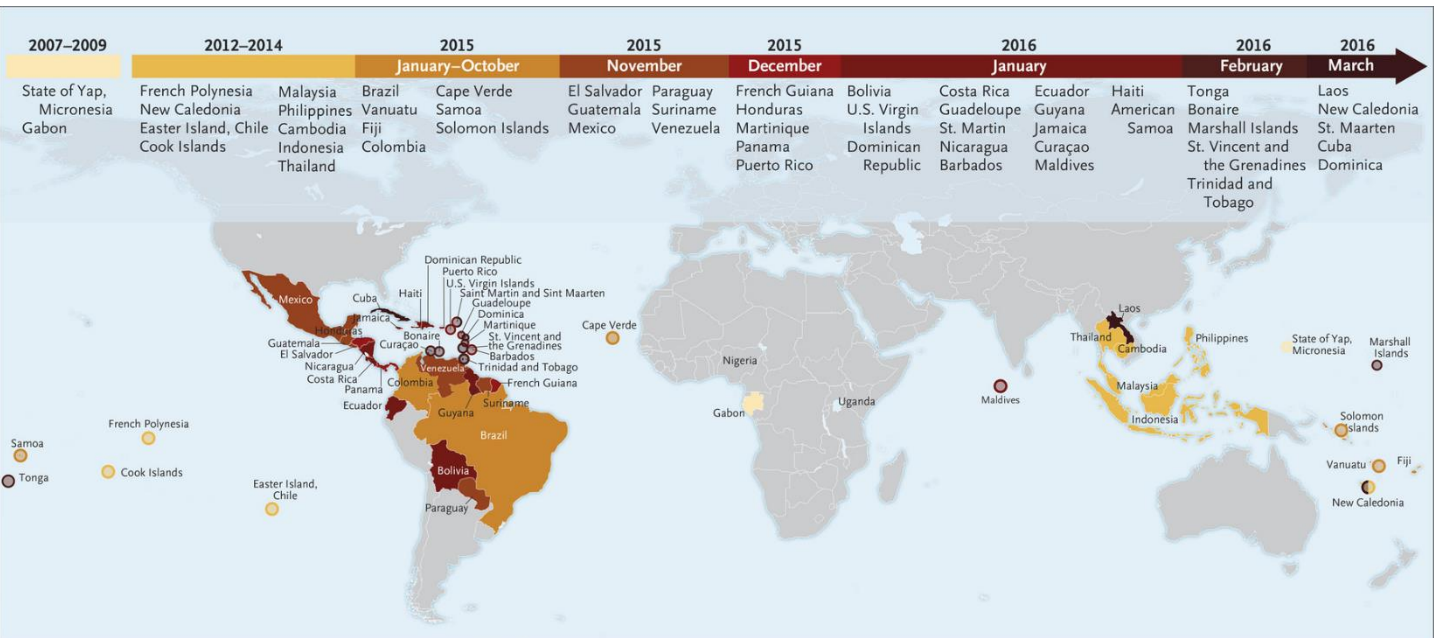
Epidemiology

Vector-borne transmission

Zika virus was discovered in Zika forest, Uganda in 1947 (72). Since 1947, a total of 14 sporadic infections in humans had been reported from Africa and Asia. These had resulted in an asymptomatic course or in mild disease only, with flu-like symptoms, viral exanthema, arthralgia and conjunctivitis. Then, in 2007 an outbreak of an Asian lineage of ZIKV was documented in Yap island, Federated States of Micronesia. It was estimated that 73% of the population of 7,391 seroconverted during the outbreak that lasted 3 months. The high attack rate indicated that Zika virus had established an urban transmission cycle. The mosquito vector involved in transmission was *Ae. hensilli* (73). The next large ZIKV epidemic occurred in French Polynesia in 2013-2014. The probable vector was another species of the *Aedes* genus, *Ae. polynesiensis* (74). Based on phylogenetic, epidemiological and mathematical modelling, ZIKV was probably introduced in Brazil by infected travellers from French Polynesia in 2013 (75,76). Autochthonous transmission of ZIKV by *Ae. aegypti* was detected in Brazil in May 2015, where it spread explosively. In Salvador, northeastern Brazil, seroprevalence rates exceeded 60% in 2016 (77). In February 2016, the World Health Organization (WHO) declared that the Zika outbreak in the Americas constituted a Public Health Emergency of International Concern (PHEIC), because of the risk of international spread, the unexpected and serious manifestations of ZIKV disease (see 'Non-vector borne transmission' and 'Clinical presentation'), and the implications for control beyond the affected countries that required immediate action (78). By 2017, the Pan American Health Organization had recorded more than 220,000 confirmed and 580,000 suspected cases in 52 territories throughout Latin America and the Caribbean (79). The high attack rates led to rapidly increasing herd immunity in the affected territories and to decline of the ZIKV epidemic in 2017 (77,80).

Figure 10. Global distribution of Zika virus from 2007 onwards.



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Zika virus infections acquired in areas with active transmission were also frequently imported into the USA and Europe by travellers. In 2016, health authorities (US Centers for Diseases Control (CDC) and European Center for Diseases Control (ECDC)) and global networks of travel clinics such as GeoSentinel reported 4897 cases in USA and 2077 cases in Europe. The reporting of these surveillance data is essential to health professionals and public health authorities, because international air travel is an important route for dissemination of vector-borne diseases by viremic travellers (81,82).

Travellers can also function as sentinels for the circulation of vector-borne pathogens in countries that are underreporting ongoing transmission of disease. However, surveillance reports are likely to underestimate the risk of travel-associated infection, because not all infections are reported to these clinic-based surveillance systems, and because of the absence of denominator data.

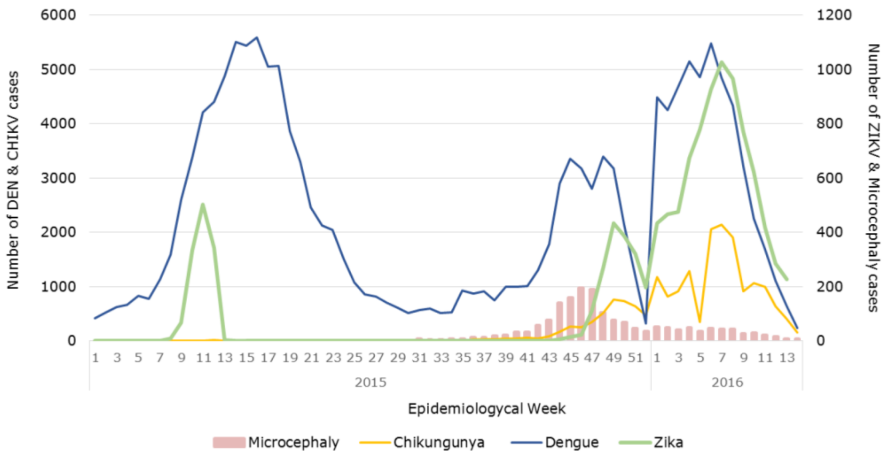
Non-vector borne transmission

A novelty in *Flaviviridae* epidemiology is person-to-person transmission of ZIKV, other than transmission by transfusion of infected blood products or laboratory accidents.

Mother-to-fetus transmission was first suspected when the Brazilian Ministry of Health issued an epidemic alert about the increase of microcephaly cases in State of Pernambuco, northeastern Brazil in November 2015 (83). This observation was epidemiologically correlated to the ZIKV outbreak in this state, 7 months earlier (Fig. 11). Evidence in support of mother-to-fetus transmission, and a causal association of maternal ZIKV infection with congenital birth defects accumulated in the following months: fetal imaging studies, the detection of ZIKV-RNA by RT-PCR in amniotic fluid of infected mothers and in fetal tissues were reported (84).

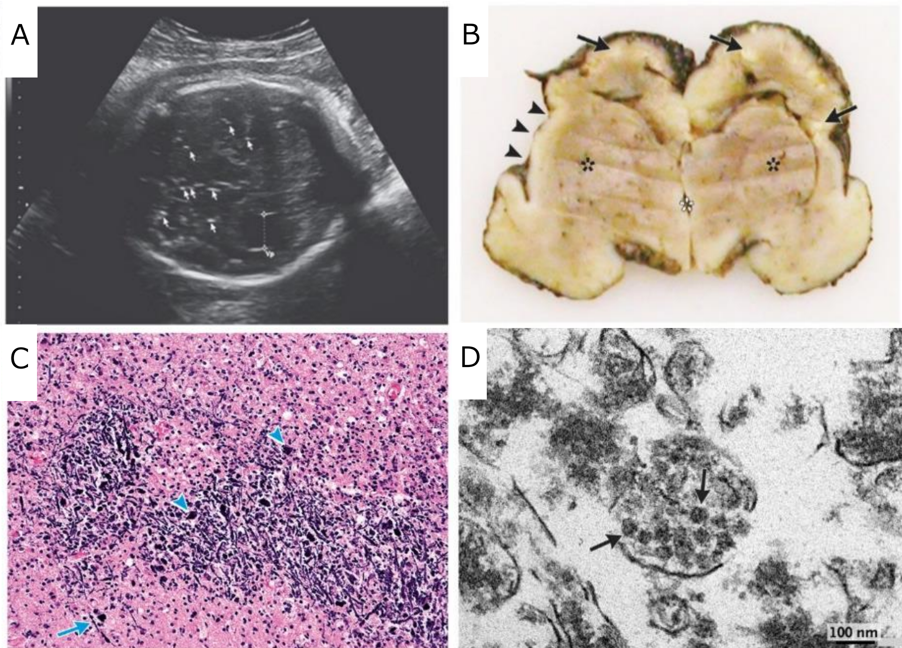
Ultimately, after the termination of the pregnancy of an infected Slovenian traveller who returned from Brazil, at fetal autopsy the virus was visualized by electron microscopy in and ZIKV was isolated from the brain tissue (Fig. 12). The complete genome sequence was recovered and matched that of the epidemic strain in Brazil (85).

Figure 11. Epidemiological correlation of microcephaly with Zika virus outbreak in Pernambuco, Brazil.



Source: Data published by the Pernambuco State Secretary of Health, Brazil.

Figure 12. Evidence that Zika Virus is associated with microcephaly.



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Probable sexual transmission of ZIKV was suggested in a case report from a scientist with serologic evidence of ZIKV infection, who infected his wife in the USA after returning from field work in Senegal (86). Definite proof of sexual transmission was presented in 2016, when the phylogenetic analysis of the ZIKV genome sequence obtained from the semen of an index patient matched that of ZIKV isolated from his sex partner' s saliva (87). Because the route of infection - vector-borne or sexual- cannot be distinguished in endemic areas, many cases of sexual transmission of ZIKV were documented in returning travellers. The majority of publications involved male-to-female sexual intercourse, but female-to-male and male-to-male transmission was also reported (88). The observed rates of sexual transmission were only 1% among returning travellers (82). The contribution of sexual transmission to the basic reproduction number (Ro), which is the average number of secondary cases resulting from the introduction of a single infectious individual in a completely susceptible population, is probably small for ZIKV. Sexual transmission of ZIKV was estimated to account for less than 3% of overall transmission rates of ZIKV (89). Still, the possibility of infecting their sexual partners late after travel-associated infection or unknowingly after asymptomatic infection, is of great concern to returning travellers.

Fig. 12, A-D., Legend (opposite page)

Panel A: prenatal ultrasonographic image showing numerous calcifications in various parts of the brain (some marked with arrows) and the dilated occipital horn of the lateral ventricle; Panel B: Photograph of coronal slice of the brain showing multifocal cortical and subcortical white calcifications (arrows) and almost complete loss of gyration of the cortex. The basal ganglia are developed but poorly delineated (black asterisks), and the sylvian fissures are widely open on both sides (arrowheads on the left). The third ventricle is not dilated (white asterisk). Panel C: magnification of calcifications with filamentous structures (arrow), possibly representing encrusted, damaged axons and dendrites, and oval and polygonal structures (arrowheads), possibly representing encrusted, damaged neuronal-cell bodies (hematoxylin and eosin staining; Panel D (lower) shows Electron Microscopy image of ultrathin sections of the fetal brain with virions clearly visible (arrows).

Clinical presentation

In the French Polynesian and American outbreaks, ZIKV infection caused symptoms in humans 3 to 7 days after the bite of an infectious mosquito (68,90). Asymptomatic infections are frequent. Based on household surveys in Yap, only 19% of ZIKV-IgM positive cases reported a clinical illness attributable to ZIKV infection (73). Seroprevalence studies estimated the rate of asymptomatic infections in French Polynesia at 50% (74). Symptomatic cases develop fever, rash, conjunctivitis, headaches, muscle or joint pain and fatigue (7,68). The clinical presentation is generally mild, but severe illness with multi-organ failure, thrombocytopenia and bleeding, or meningo-encephalitis in adults has been reported (91–93). Infection of the male reproductive tract occurs frequently, and leads to oligospermia and affect the metabolism of sex hormones (94). The consequences of infection for male fertility need further study.

During the outbreak in French Polynesia, ZIKV infection was identified as a trigger of Guillain-Barre Syndrome, increasing the risk of this post-infectious and immune-mediated peripheral neuropathy up to 30 times (95,96). However, it has not been reported consistently and tends to occur more in some geographical areas than in other. Further evidence is needed to determine whether the virus acts alone, or to establish the role of environmental and host (such as previous dengue virus infection) as potential co-factors (97).

The range of neurological disorders in neonates who were born to mothers infected with ZIKV during of pregnancy (all trimesters), represents the most devastating consequences of ZIKV disease. The defects and abnormalities, that can be manifest in fetal life and at birth or that may be detected during later developmental stages, are referred to as Congenital Zika Syndrome (CZS) (98,99). They include structural brain and eye anomalies at birth, such as microcephaly, which is defined as a head circumference more than 2 SD below the mean for infant sex and gestational age, intracranial calcifications and ventriculomegaly (Fig 13. A, B and C), but also hearing and visual impairment, multiple contractures (arthrogryposis) (Fig. 13 D), body tone and movement abnormalities, seizures and postnatal-onset microcephaly (99,100).

Birth defects associated with CZS were detected in 4-8% of pregnancy outcomes of mothers with confirmed ZIKV infection (101–105). In returning pregnant travellers, the US Zika Pregnancy Registry reported a similar attack rate of CZS in symptomatic and asymptomatic maternal ZIKV infections (102–104). The risk of CZS was highest when maternal infection occurred in the first trimester of pregnancy (104,105). The full spectrum of CZS and its detrimental impact on the cognitive and neurological development of the first generation of affected children is investigated intensely in large cohorts with long-term follow-up across Latin America (106).

Figure 13. Clinical findings in congenital Zika syndrome.

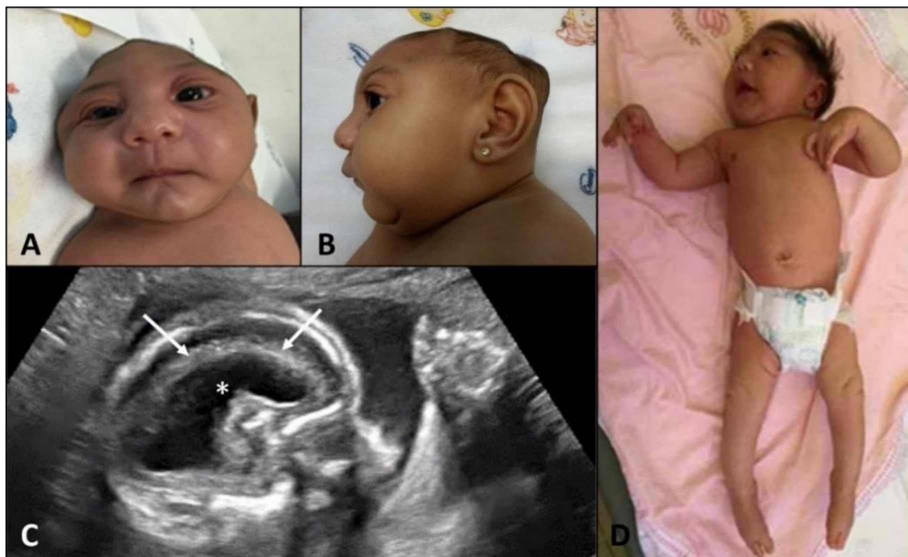

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Fig. 13 Panel A and B. The head of an infant with congenital Zika virus infection (front and lateral view). The frontal view shows severe loss of cranial height and depressed frontal bones. On lateral view, frontal and parietal bones are greatly depressed because of loss of brain parenchyma. The occipital bone is prominent and excess scalp is seen at the nape of the neck. Panel C shows ventriculomegaly (*) and bandlike subcortical calcification (arrows) seen by ultrasound in a fetus at 22 weeks' gestation. Panel D: Full view of an infant with congenital Zika virus infection and microcephaly and arthrogryposis. Joints involved include bilateral contractures of the wrists, hips, knees, and ankles.

Diagnosis

ZIKV infection can be confirmed by isolation of the virus, and a range of molecular and serologic tests (107). In this section I will introduce the assays that have been used for diagnosis at ITM. As was illustrated in Fig. 8, the selection of appropriate tests to confirm arbovirus infection depends on the kinetics of the virus (or its nucleic acids or viral proteins), and of virus-specific IgM and IgG antibodies in clinical samples. At the start of the ZIKV outbreak in the Americas, neither the kinetics of ZIKV-RNA in serum or in other body fluids, nor the human immune response to infection were well characterized. Inferences were made from infection by and immune responses to better documented *Flaviviridae*, notably dengue virus, to inform 'educated guesses' about the natural history of ZIKV infection (108,109).

Data on the diagnostic performance of RT-PCR for the epidemic ZIKV strains and in different clinical sample types were scarce (110). ZIKV RNA can be detected by RT-PCR in serum from a few days prior to 5 days after symptom onset (111,112). ZIKV RNA has been detected in other body fluids, e.g. whole blood, saliva, nasopharyngeal secretions, breast milk, semen, and urine (113). ZIKV nucleic acids have been demonstrated to persist longer in whole blood, in urine and in semen. Only RT-PCR on urine (in addition to serum) has been adopted widely for the diagnosis of ZIKV, because of the ease of sampling, the observed consistency of detectability and the higher sensitivity of combined testing (114). For the studies presented in this thesis, a ZIKV-specific in-house duplex real-time RT-PCR that targeted a 102bp and 121bp sequence of the NS5 gene or the RealStar® Zika Virus RT-PCR Kit (Altona diagnostics GmbH, Hamburg, Germany) were used (110). It is important to recognize that a negative RT-PCR result in the acute phase does not rule out a diagnosis of ZIKV infection.

Detection of ZIKV-specific antibodies by ELISA or immunofluorescence assay is the preferred diagnostic method in patients who present after the acute phase. Only one description of ZIKV antibody kinetics in combined acute and convalescent human sera prior to the outbreak in

the Americas, had been published. In eleven patients, a subset from the Yap outbreak, IgM was detected by ELISA as soon as 3 days after symptom onset, suggesting that IgM could be used to identify recent ZIKV infections (111). No data was available on the persistence of ZIKV-specific IgM. In dengue virus infections, DENV-specific IgM remained detectable for three months (115). At the start of the outbreak in Brazil, the timing to IgG seroconversion in ZIKV infection was unknown. These knowledge gaps brought additional challenges to the interpretation of serologic test results during the first month of infection and the development of diagnostic algorithms (116).

In the Yap study, six of seven patients with serologic evidence of previous flavivirus infections or vaccinations, also had positive IgM-results against one or more other flaviviruses. The appearance of high titers upon exposure to heterologous flaviviruses has been described as the “original antigenic sin” (117). This phenomenon also affects IgG-class antibodies, and is more commonly observed in endemic than in travellers’ populations. The cross-reactivity has been attributed to the similarity of important antigens of flaviviruses, as is illustrated in Fig. 14 for the envelope proteins E of ZIKV and DENV serotype 2 (118). Antibodies to the nonstructural protein 1 (NS1) showed higher specificity for ZIKV (119). Early in the epidemic, an ELISA was developed and commercialized that uses recombinant ZIKV NS1-antigen for antibody capture. This assay, the anti-ZIKV IgM/IgG ELISA (Euroimmun, Lübeck, Germany), which showed high specificity for ZIKV-specific antibody detection in panels that included samples from patients with heterologous flavivirus and other infections, as well as blood donors from different geographic origins, was used in our studies (120,121).

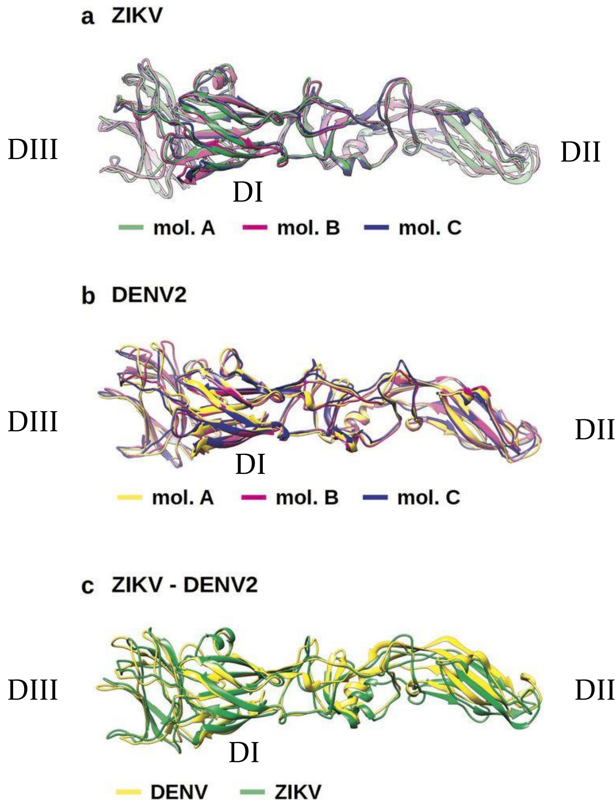
Immunofluorescence tests utilize whole virus particles in Zika virus-infected cells as the antigenic substrate. The results are evaluated by fluorescence microscopy. The immunofluorescence assays have higher sensitivity than the antigen-based ELISA. The trade-off is that cross-reactivity between IgG antibodies against heterologous flaviviruses are more likely to occur and specificity of these assays is therefore generally

lower. We used the Euroimmun Anti-Zika Virus IgM Indirect Immunofluorescent test (IIFT) to evaluate samples with equivocal IgM results in ELISA, or samples with neg IgM and positive IgG ELISA results from patients with recent symptom onset (up to 8 weeks).

Neutralization tests can be used to confirm the specificity of cross-reactive antibodies against arboviruses detected by ELISA or immunofluorescence assay (111,122). To establish if a serum sample contains neutralization antibodies to viruses, a virus-infected cell monolayers is inoculated with the patient's serum and the reduction of virus-induced cytopathic effects after a set incubation period is quantified. Because this is a laborious and costly procedure, it is not frequently employed in a routine diagnostic setting.

The choice of tests also depends on the availability of validated diagnostic assays. At the start of the ZIKV outbreak in the Americas, only a very limited number of molecular and serological tests had been licensed for commercial use (116). None of these had been validated against the epidemic strains.

Fig 14. Comparison of envelope proteins of ZIKV and DENV Serotype 2



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The envelope protein (E) of Flaviviridae is the major protein involved in receptor binding and fusion. Envelope and membrane proteins are organized in icosahedral symmetry consisting of 60 repeating units, and each asymmetric unit contains three individual E proteins, molecules A, B and C. The E ectodomain contains three domains: DI, DII and DIII (see also Fig. 9). Superposition of the three individual molecules A, B and C (using DI as a reference point) that make up the ectodomains of protein E, show high similarities between them for ZIKV (Fig. 14a) and for DENV2 (Fig. 14b). The superposition of molecule A of ZIKV (green) with that of DENV2 (yellow) by their DIs in Fig 14c. shows that they have slightly different DI–DIII and DI–DII hinge angles, but illustrates the high similarity between these antigens. The cross-reactivity that is observed in anti-flavivirus antibody detection assays has been attributed to the similarity of important antigens of flaviviruses.

Identification of knowledge gaps

The general objective of this thesis is to examine the risk factors, the clinical presentation and diagnostic challenges of CHIKV and ZIKV infections, and to reflect on their implications for case management.

To tailor appropriate clinical management for individual CHIKV-infected patients, it is essential to be able to identify predictors of chronic arthralgia and to confirm the diagnosis during the acute phase of infection. Predictors of long-term sequelae of CHIKV infection have been identified in previous outbreaks and include age (>35-50 years), gender (female), and pre-existing comorbidities (6). Data on the persistence of joint pains after infection with the Asian genotype CHIKV that emerged in the Americas in 2013 and following years was not available. A rapid point-of-care test to detect CHIKV antigen is not commercially available. Such a direct method would likely have an impact on the clinical management of CHIKV-infected patients, similar to the introduction of rapid diagnostic tests for detection of the nonstructural 1 (NS1) dengue antigen for dengue patients (123,124).
The availability of a large cohort of patients with suspected CHIKV infection from Aruba, an island in the Dutch Caribbean, gave us the opportunity to address these knowledge gaps, following the first outbreak of an Asian genotype CHIKV in Aruba in 2014-2015.

In 2015, the emergence of the previously obscure ZIKV and its unexpected complications in the Americas challenged the international scientific community and leading public health authorities with important knowledge gaps in the diagnosis and management of this infection. Dr. Margaret Chan, then Director-General of the World Health Organization, responded to the threat of rapid, international spread of ZIKV by declaring the outbreak a Public Health Emergency of International Concern (PHEIC) on February 1, 2016 (125).

To control and prevent ZIKV (and other arboviral infections like CHIKV and dengue virus), her advising Expert Committee made recommendations that included the rapid development of diagnostics and improved communication about the risks of outbreaks of ZIKV and other arboviruses (78).

Travellers' concerns were most tangible in the consultation rooms of travel clinics, where physicians performed a daunting task of risk communication to outbound travellers or the diagnostic evaluation of persons exposed to or infected with ZIKV in the absence of validated testing algorithms (109,116,122,126,127).

Research objectives of this thesis

1. Retrospective confirmation of the diagnosis of CHIKV infection by real-time reverse transcription polymerase chain reaction (RT-PCR) in stored sera, obtained during the acute phase of illness in a cohort of patients with suspected CHIKV infection.

2. Evaluation of the contribution of an antibody detection assay and nucleic acid detection by RT-PCR to the composite diagnosis of chikungunya virus infection in the acute phase.

3. Detailed assessment of clinical symptoms and identification of clinical predictors of post-chikungunya chronic poly-arthralgia in confirmed cases.

4. Evaluation of the diagnostic accuracy of a prototype immuno-chromatographic test for the detection of CHIKV E1-antigen at the Institute of Tropical Medicine in Antwerp (ITM).

5. Determination of the incidence rate of ZIKV infection in Belgian travellers, who were recruited before visiting areas with active vector-borne transmission of ZIKV during the outbreak in the Americas in 2016.

6. Detailed assessment of the frequency and kinetics of ZIKV infection in semen in returning Belgian travellers with confirmed ZIKV infection.

7. Detailed assessment of the presence of clinical signs and symptoms of ZIKV infection in returning travellers who were evaluated for ZIKV infection at ITM, during the outbreak in the Americas.

8. Assessment of the clinical utility of a newly developed diagnostic algorithm to rule out recent ZIKV infection by calculation of the post-test probability for a negative ZIKV-specific antibody detection result in asymptomatic travellers.

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Chapter 1

Chikungunya virus infection

and chronic joint pains

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Chikungunya virus infection in Aruba: diagnosis, clinical features and predictors of post-chikungunya chronic polyarthralgia

Huits R.[1¶*], De Kort J.[2¶], Van Den Berg R.[3,#a], Chong L.[3,#a], Tsoumanis A.[1], Eggermont K.[1], Bartholomeeusen K.[4], Ariën K.K.[4,5], Jacobs J.[1,6], Van Esbroeck M.[1], Bottieau E.[1&], Cnops L.[1&]

[1] Department of Clinical Sciences, Institute of Tropical Medicine, Antwerp, Belgium
[2] Department of Internal Medicine, Horacio Oduber Hospital, Oranjestad, Aruba
[3] Landslaboratorium Aruba, Oranjestad, Aruba
[4] Department of Biomedical Sciences, Institute of Tropical Medicine, Antwerp, Belgium
[5] Department of Biomedical Sciences, University of Antwerp, Antwerp, Belgium
[6] Department of Microbiology and Immunology, University of Leuven, Leuven, Belgium

[#a] Current address: Department of Public Health, Oranjestad, Aruba
[¶&] These authors contributed equally to this work.

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Abstract

Background

Chikungunya virus (CHIKV) emerged in Aruba for the first time in 2014. We studied the clinical presentation of acute CHIKV infection and the contribution of serologic and molecular assays to its diagnosis. In a cohort of confirmed CHIKV cases, we analysed the frequency, duration and predictors of post-chikungunya chronic polyarthralgia (pCHIK-CPA), defined as joint pains lasting longer than 6 weeks or longer than 1 year.

Methods

Patient sera obtained within 10 days of symptom onset were tested for CHIKV, using an indirect immunofluorescence test for the detection of CHIKV-specific Immunoglobulin M (IgM) and post-hoc, by reverse-transcription polymerase chain reaction (RT-PCR). CHIKV was isolated from selected samples and genotyped. For confirmed CHIKV cases, clinical data from chart review were complemented by a telephone survey, conducted 18-24 months after diagnosis. When joint pain was reported, the duration, presence of inflammatory signs, type and number of joints affected, were recorded. Joint involvement was scored according to the 2010 'American College of Rheumatology/ European League Against Rheumatism' criteria for seronegative rheumatoid arthritis (ACR-score). Risk factors for pCHIK-CPA were identified by logistic regression.

Principal findings

Acute CHIKV infection was diagnosed in 269 of 498 sera, by detection of IgM (n=105), by RT-PCR (n=59), or by both methods (n=105). Asian genotype was confirmed in 7 samples. Clinical data were complete for 171 of 248 (69.0%) patients, aged 15 years or older (median 49.4 [35.0 - 59.6]). The female-to-male ratio was 2.2. The main acute symptoms were arthralgia (94%), fever (85%), myalgia (85%), headache (73%) and rash (63%). In patients with arthralgia (n=160), pCHIK-CPA longer than 6 weeks was reported by 44% and longer than 1 year by 26% of cases.

Inflammatory signs, stiffness, edema and redness were frequent (71%, 39% and 21%, respectively). Joints involved were knees (66%), ankles (50%), fingers (52%), feet (46%), shoulders (36%), elbows (34%), wrists (35%), hips (31%), toes (28.1%) and spine (28.1%). Independent predictors of pCHIK-CPA longer than 1 year were female gender (OR 5.9, 95%-CI [2.1-19.6]); high ACR-score (7.4, [2.7-23.3]), and detection of CHIKV-RNA in serum beyond 7 days of symptom onset (6.4, [1.4-34.1].

Conclusions

We identified 269 CHIKV patients after the first outbreak of Asian genotype CHIKV in Aruba in 2014-2015. RT-PCR yielded 59 (28%) additional CHIKV diagnoses compared to IgM antibody detection alone. Arthralgia, fever and skin rash were the dominant acute phase symptoms. pCHIK-CPA longer than 1 year affected 26% of cases and was predicted by female gender, high ACR-score and CHIKV-RNA detection beyond 7 days of symptom onset.

Introduction

The word 'chikungunya' was used by the Makonde people of Southern Tanzania to describe the severe joint pains that literally bent the affected patients' posture. The causative agent, chikungunya virus (CHIKV), was first isolated during an explosive outbreak in East Africa in 1952 (1,2). It is a positive-sense single stranded RNA virus that belongs to the genus *alphavirus* of the family *Togaviridae*. The virus is transmitted by mosquitoes, and *Aedes aegypti* was identified as the primary vector (3). Three genotypes of CHIKV have been identified: West African, Eastern/Central/Southern African (ECSA) and Asian (4).

After the isolation and description of CHIKV, spill-over infection from sylvatic transmission cycles and small scale epidemics were reported from African and South-East Asian countries in the second half of the twentieth century (5). However, since 2005 ECSA and Asian genotype CHIKV has spread across continents in outbreaks that involved millions of people and put millions more at risk globally (6,7). The emergence of CHIKV as a global pathogen has been attributed to multiple factors. Urbanization in countries where CHIKV was endemic, allowed convergence of human and vector populations. Increased air travel permitted frequent exposure and rapid spread of susceptible human populations to the virus. International trade, climate change and lack of adequate vector control measures contributed to suitable environments for geographic expansion of its vector species (8). Finally, showcasing the nature of evolution, CHIKV adapted to replication in *Aedes albopictus* by a single mutation in the envelope protein gene (E1-A226V) in the ECSA genotype. The resulting increase in infectivity of this highly competent vector led to enhanced transmission during the 2005-2006 outbreak on Reunion island (9).

Signs of acute CHIKV infection other than arthralgia, are fever, myalgia and rash. Clinical distinction from other arthropod-borne viral illness is not possible. Still, diagnosing CHIKV infection at an early stage is important, as the acute febrile illness is frequently followed by post-chikungunya chronic polyarthralgia (pCHIK-CPA). The chronic,

symmetric joint pains of pCHIK-CPA may resemble seronegative rheumatoid arthritis (10,11).

Aruba is an island in the Lesser Antilles of 180 square kilometres, north of Venezuela. Its population of approximately 100,000 inhabitants is of mixed descent, originating from Europe, Latin America, and Africa (12).

Our study of the first CHIKV outbreak in Aruba had three objectives. First, we evaluated the contribution of serologic and molecular testing to the diagnosis of CHIKV in the acute phase. Second, we performed a retrospective assessment of the clinical presentation of CHIKV infection in adults. Third, we identified risk factors associated with pCHIK-CPA.

Methods

During the 2014-2015 CHIKV outbreak, Aruban patients were evaluated for CHIKV at their physician's discretion, using a serum sample obtained within 10 days post symptom onset (DPSO) (Fig 1). The laboratory diagnosis of acute CHIKV infection in a single sample requires a testing algorithm that combines an assay for CHIKV-specific immunoglobulin M (IgM) antibody detection and a molecular method for CHIKV-RNA detection (13,14).

Diagnostics

Acute CHIKV infection in our study was defined by either or both positive results of CHIKV-specific IgM in an IgM/IgG indirect immunofluorescence assay (IFA) (Anti-CHIKV IIFT IgM/IgG, Euroimmun, Lübeck, Germany) according to manufacturer's instructions, and CHIKV-specific real-time reverse-transcription polymerase chain reaction (RT-PCR). Sera with positive IgG, but negative IgM result in IFA and negative RT-PCR were not considered acute infections. Convalescent sera to assess seroconversion were not available. The Landslaboratorium was the only diagnostic facility on the island that tested for CHIKV, using IFA. All sera were stored at the Landslaboratorium at -80°C, until shipment to the Institute of Tropical Medicine (ITM, Antwerp Belgium), where CHIKV-specific RT-PCR was

performed as described previously. Briefly, a specific part of the nonstructural protein 1 (NSP-1) gene was amplified on the LightCycler 96 (Roche) according to the protocol described by Van den Bossche et al., with primers and probes detecting the African and Asian CHIKV strains (15,16). Real-time RT-PCR was run for 50 cycles, and any Ct-value < 50 was considered positive. Isolation of CHIKV by inoculation onto Vero cells (ATCC® CCL-81™) was attempted from samples with Ct-values below 30. The E1-region of the isolates was genotyped (Sanger sequencing method) (17).

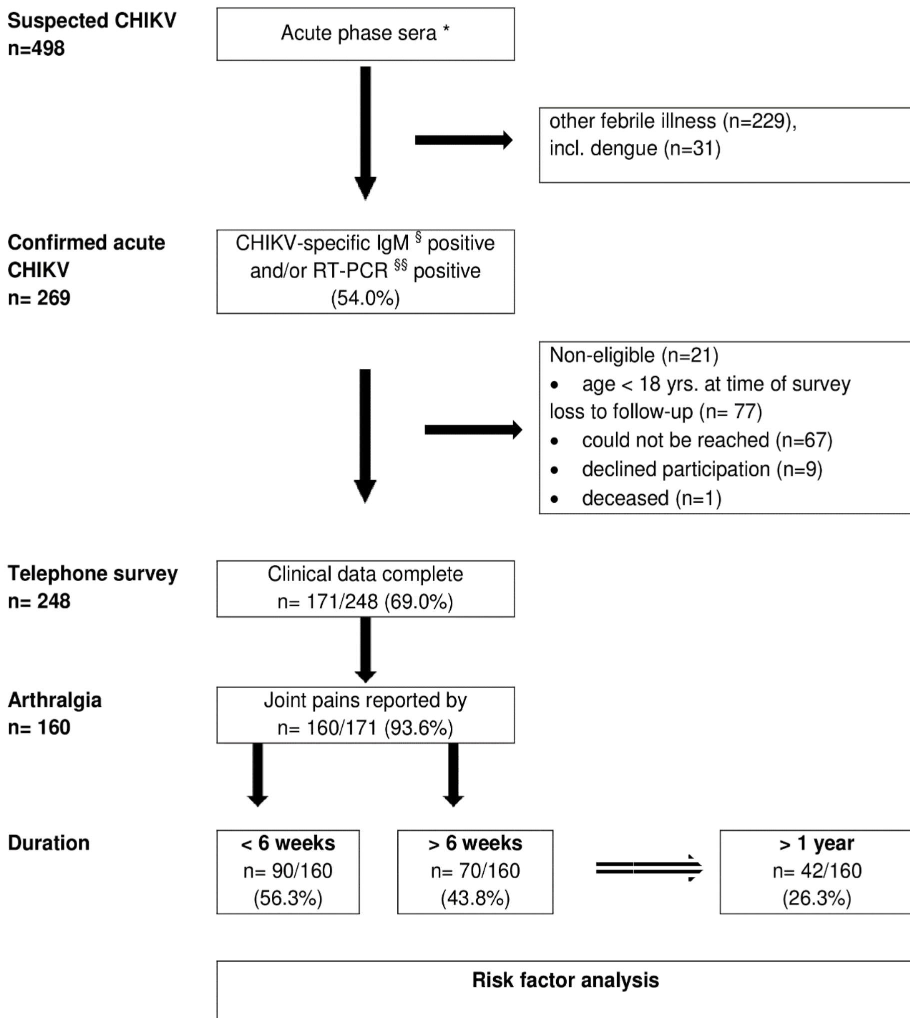
Because dengue virus (DENV) is endemic in Aruba, a DENV multiplex RT-PCR to differentiate the four serotypes (DENV1-4) was performed with an in-house test (adapted from ref. (18)) on the sera of confirmed acute CHIKV cases, to exclude co-infection.

Data collection

Clinical data from the laboratory request forms (gender, age and date of symptom onset- defined as 0 DPSO) were complemented by a structured telephonic questionnaire. The interviews were conducted by a trained infectious disease physician, 18-24 months after diagnosis, after obtaining oral consent. Patients with confirmed CHIKV aged 18 years or older, were eligible for interviewing. Participants were excluded after three failed attempts to contact them. Additional data obtained included comorbidities (diabetes, obesity, history of cardiovascular, respiratory or pre-existing rheumatological conditions, osteoporosis, osteoarthritis) and symptoms in the acute phase (fever, rash, arthralgia, myalgia, headache, respiratory or gastro-intestinal symptoms, difficulty sleeping or concentrating).

When arthralgia was reported, the pattern of joint involvement was systematically scored in accordance with the 2010 American College of Rheumatology/ European League Against Rheumatism (ACR/EULAR) criteria for seronegative rheumatoid arthritis (19). Briefly, the pattern of distribution of affected joints was scored based on the number and type of joints involved. 'Large joints' refers to shoulders, elbows, hips, knees, ankles and spine; 'Small joints' refers to the metacarpophalangeal, interphalangeal, metatarsophalangeal and wrist joints.

Fig 1. Study design.



** Acute phase sera refers to all available sera collected within 10 days of symptom onset. § Positive IgM: Immunoglobulin M (IgM) detected in indirect immunofluorescence assay (IFA) (Anti-CHIKV IIFT IgM/IgG, Euroimmun, Lübeck, Germany) according to manufacturer's instructions. §§ positive RT-PCR: CHIKV RNA detected in a RT-PCR targeting the NSP-1 gene using primers and probes that detect CHIKV strain (LR2006_OPY_1, Indian Ocean Islands and the African prototype (S27, general strain)).*

We assigned low ACR-scores '1, 2 and 3' to 'one large joint, 2-10 large joints, and 1-3 small joints (with or without involvement of large joints), respectively. High ACR scores '4 and 5' were assigned when involvement of '4-10 small joints (with or without involvement of large joints) or involvement of more than 10 joints (involving at least one small joint)' were recorded. Symmetry, defined as bilateral involvement of at least one joint region, was assumed in all cases, unless participants explicitly reported involvement of a single joint (11). Reported edema, redness and morning stiffness that lasted more than 30 minutes were recorded as clinical signs of inflammatory arthropathy. Self-reported duration of pain in adults with confirmed CHIKV was scored in six categories: shorter than 2 weeks, 2-6 weeks, 6 weeks-3 months, 3-6 months, 6-12 months or longer than 12 months. pCHIK-CPA was defined as joint pain lasting more than 6 weeks.

The frequency of medical consultations and duration of absenteeism (less than one week, 1-2 weeks, 2-4 weeks, more than 4 weeks or still not able to work) were recorded.

Outcomes

As primary outcomes, we recorded the relative contribution of RT-PCR and IgM antibody assays to the compound diagnosis of acute CHIKV infection per day of sampling post symptom onset, the duration of pain in adults with confirmed CHIKV (shorter than 6 weeks, longer than 6 weeks and longer than one year) and potential risk factors for pCHIK-CPA, lasting more than 6 weeks or more than 1 year. Secondary outcomes were the frequency of follow-up medical consultations and the duration of absenteeism.

Statistics

Continuous variables were summarized as medians and inter-quartile ranges. Categorical variables were expressed as counts and percentages. Chi-square test or Fisher's exact test were used to test for association between categorical variables. Odds ratios (OR) with 95%-CI were calculated to identify risk factors in univariate logistic regression models. When significant at 10% level, a multivariate regression model

was fitted to adjust for confounding and multiple predictors. The final model was selected using stepwise backward elimination with likelihood ratio test as the comparison test. All analyses were done in R (version 3.4.1).

Ethics statement

Ethics approval for this study was obtained from the Institutional Review Board at the Institute of Tropical Medicine and Ethics Committee of the University Hospital in Antwerp, Belgium. Approval for the collection of clinical data by telephonic survey after oral consent was obtained from the Board of the Horacio Oduber Hospital in Aruba. All subjects were 18 years or older when interviewed by a physician, who proceeded only after oral consent and documenting the obtained consent on the questionnaire form.

Results

Sera of 498 patients with clinical suspicion of acute CHIKV infection were collected within the first 10 DPSO, from October 2014 (epiweek 42) to April 2015 (epiweek 11) (Fig 2). In total, acute CHIKV infection was diagnosed in 269 (54.0%) patients, 175 female and 94 male (female-to-male ratio 1.9).

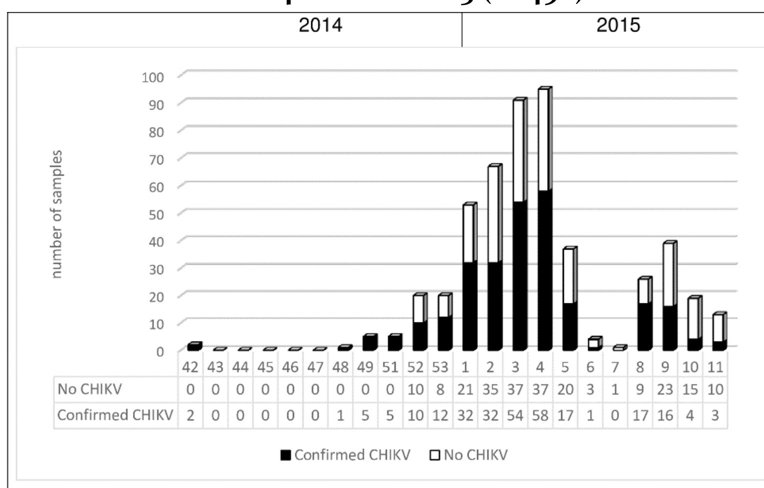
CHIKV-specific IgM was positive in 210 patient sera, in 105 of which RT-PCR was also positive. In addition, RT-PCR was positive in 59 sera for which CHIKV-specific IgM was negative. CHIK-specific IgG was detected in 122 of the 210 patients with positive IgM result, and in 56 of the 164 RT-PCR positive samples. CHIKV-specific IgG alone was detected in 9 out of 498 sera (1.8%), all collected from January 2015 onwards.

The exact date of sampling post symptom onset was available for 261 out of 269 sera (97.0%) with confirmed CHIKV infection and the respective proportions of positive IFA and RT-PCR results was calculated on this number (Fig 3.). RT-PCR was still positive in up to 50% of the sera collected between DSPO 8-10.

DENV-RNA was not detected in any of the 269 sera with confirmed acute CHIKV. In 31 of 229 sera without CHIKV (13.5%), evidence of acute

DENV infection was found. DENV-specific RT-PCR was positive in 6, and DENV-specific IgM was positive in 25.
Isolation of CHIKV by inoculation onto Vero cells was attempted from 13 samples with Ct-values ranging from 17.3 to 31.0, and was successful in 8 (Ct-values 17.3-28.7). The E1-region (position 9994-11310) of 7 isolates was genotyped by Sanger sequencing; all 7 clustered with the Asian-Caribbean strain.

Fig. 2. Number of samples collected for chikungunya diagnostics per epidemiological week, during the outbreak in Aruba from October 2014 – March 2015 (n=498).

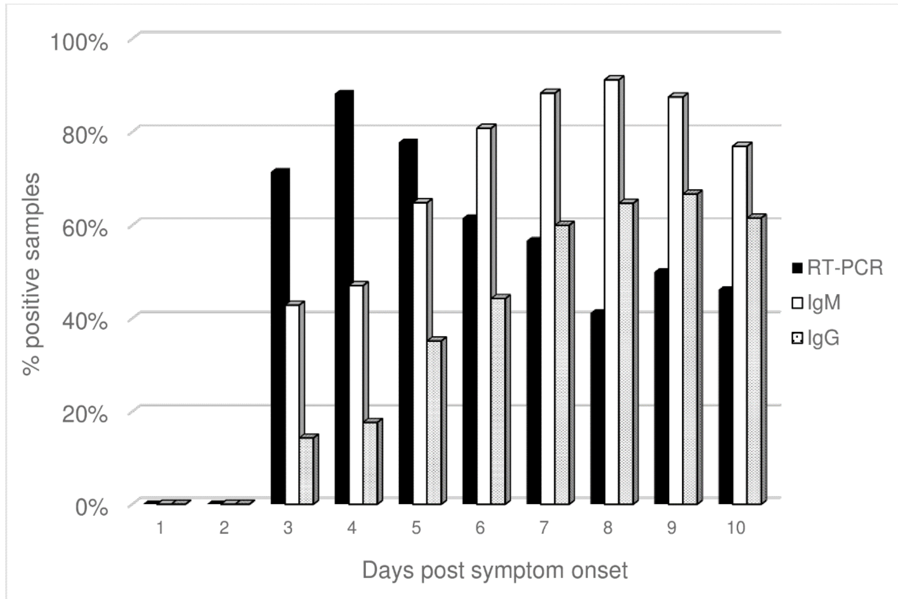


Note: epiweek 6 and 7 coincided with Carnival, Aruba's public holiday.

Follow-up interviews were conducted from June 2016 to June 2017. At that time, 21 of 269 confirmed CHIKV cases were younger than 18 years, leaving 248 eligible for clinical reassessment by telephonic survey. Hundred seventy-one of 248 patients (69.0%) were interviewed. Sixty-seven were not contacted after 3 attempted calls, 9 declined to be interviewed, and one had died of causes unrelated to CHIKV infection. Their median age was 49.4 years (IQR 35.0- 59.6 years). Hundred seventeen respondents were female, 54 were male (female-to-male ratio 2.2). The non-respondents had comparable distribution of age (p-value = 0.12, Chi-square test) and gender (p-value = 0.34, Mann-Whitney U test). Self-reported comorbidities among 171 respondents are reported in Table 1.

Fig 3. Proportions of positive chikungunya virus-specific RT-PCR, IgM and IgG antibodies (IFA) per day of sampling post symptom onset. *

Samples (n)	0	0	7	17	54	52	60	34	24	13



DPSO	3	4	5	6	7	8	9	10
RT-PCR pos, n (%)	5 (71.4)	15 (88.2)	42 (77.8)	32 (61.5)	34 (56.7)	14 (41.2)	12 (50.0)	6 (46.2)
IgM pos, n (%)	3 (42.9)	8 (47.1)	35 (64.8)	42 (80.8)	53 (88.3)	31 (91.2)	21 (87.5)	10 (76.9)
RT-PCR pos AND IgM pos, n (%)	1 (14.3)	6 (35.3)	23 (42.6)	22 (42.3)	27 (45.0)	11 (32.4)	9 (37.5)	3 (23.1)
RT-PCR pos AND IgG pos, n (%)	0	1 (5.9)	5 (9.3)	8 (15.4)	18 (30.0)	6 (17.6)	4 (16.7)	2 (15.4)

** Note: exact date of sampling was available for 261 patients with confirmed CHIKV infection.*

Symptoms during the acute phase confirmed a pattern of CHIKV infection as an acute febrile illness with arthralgia and myalgia as the most prominent clinical features (Table 1.). None of the CHIKV-infected patients were hospitalized and no CHIKV-related mortality was recorded.

Arthralgia was reported by 160 (93.6%) patients. Among these 160 patients, joint involvement was symmetrical in 156 (97.5%). The affected joints are listed in Table 2.

Seventy-eight patients (48.8%) were assigned a low ACR-score and 82 patients (51.3%) had a high ACR-score. Involved joints were stiff, edematous and red in 71.3%, 38.8% and 21.3%, respectively (Table 2.).

pCHIK-CPA longer than 6 weeks was recorded in 70/160 (43.8%) and longer than 1 year in 42/160 (26.3%) of cases. Adjusted risk factors from the multivariate analysis for pCHIK-CPA longer than 6 weeks were female gender (OR 3.4, 95%-CI [1.6-8.0]), obesity (4.8, [1.8-14.7]) and concentration disorder (4.7, [2.1-11.1]). Independent risk factors for pCHIK-CPA longer than 1 year were female gender (5.9, [2.1-19.6]), and a high ACR-score (7.4 [2.7-23.3]). For patients tested at 8, 9 or 10 DPSO (n=42), the OR for pCHIK-CPA longer than 6 weeks was 4.0 ([1.1-17.6]) , and 6.4 ([1.4-34.1]) for pCHIK-CPA longer than 1 year when RT-PCR result was positive for CHIKV-RNA, compared to patients with a negative RT-PCR beyond 7 DPSO (Table 3.).

Patients with pCHIK-CPA longer than 6 weeks or longer than 1 year consulted their physician more frequently (OR 1.8, [1.0- 3.5] and 2.2, [1.0-4.8], respectively). Hundred-and-one out of 171 interviewed patients (59.1%) reported absenteeism. Ninety-five out of 101 (94.1%) who took sick leave reported arthralgia. Four patients without arthralgia were absent from work for less than a week, two less than 2 weeks. Absenteeism longer than 2 weeks was recorded in 27.1% of patients with pCHIK-CPA longer than 6 weeks and in 31.0% of those with pCHIK-CPA longer than 1 year. Compared to patients without pCHIK-CPA, the OR for sick leave more than 2 weeks was 3.8 ([1.5-10.1]) when joint pains lasted more than 6 weeks, and 4.6 ([1.6-13.1]) when joint pains lasted more than one year.

Table 1. **Characteristics of 171 patients with confirmed chikungunya infection**

Age (years), median [IQR]	49.4	[35.0 - 59.6]
< 18 yrs.	11	(6.4)
18 – 40 yrs.	40	(23.4)
41 – 60 yrs.	78	(45.6)
> 60 yrs.	42	(24.6)
Gender	n	(%)
Female	117	(68.4)
Male	54	(31.6)
Female-to-male ratio	2.2	

Medical history	n	(%)
Diabetes	14	(8.2)
Obesity	25	(14.6)
Cardiovascular	20	(11.7)
Respiratory	11	(6.4)
Rheumatological	4	(2.3)
Osteoporosis	5	(2.9)
Osteoarthritis	11	(6.4)
Other	38	(22.2)

Symptoms	N	(%)
Arthralgia	160	(93.6)
Fever	146	(85.4)
Myalgia	145	(84.8)
Headache	124	(72.5)
Skin rash	107	(62.6)
Respiratory symptoms §	35	(20.5)
Gastro-intestinal symptoms §§	52	(30.4)
Dizziness	48	(28.1)
Bleeding	4	(2.3)
Sleeping disorder	59	(34.5)
Concentration disorder	39	(22.8)

Absenteeism	n	(%)
< 1 week	41	(24.0)
1-2 weeks	33	(19.3)
2-4 weeks	18	(10.5)
> 4 weeks	8	(4.7)
Still not able to work	1	(0.6)
Health visit (physician)	82	(48.0)

Table 2. Pattern of joint involvement, inflammatory symptoms and pain duration in chikungunya virus infected patients presenting with arthralgia (n=160)

Affected joints	n	(%)
shoulder	57	(35.6)
elbow	54	(33.8)
wrist	56	(35.0)
finger	83	(51.9)
hip	49	(30.6)
knee	105	(65.6)
ankle	80	(50.0)
feet	73	(45.6)
toes	45	(28.1)
spine	45	(28.1)

Inflammatory symptoms	n	(%)
redness	34	(21.3)
edema	62	(38.8)
stiffness	114	(71.3)

Duration of joint pains	n	(%)
< 2 weeks	59	(36.9)
2- 6 weeks	31	(19.4)
6 weeks - 3 months	11	(6.9)
3- 6 months	8	(5.0)
6- 12 months	9	(5.6)
> 12 months	42	(26.3)

ACR-score #			n	(%)
low	1.	(1 large joint)	4	(2.5)
	2.	(2-10 large joints)	42	(26.3)
	3.	(1-3 small joints)	32	(20.0)
high	4.	(4-10 small joints)	43	(26.9)
	5.	(> 10 joints)	39	(24.4)

The ACR-score was assigned according to type and number of joints involved,based on the 2010 'American College of Rheumatology/ EUropean League Against Rheumatism (EULAR)' criteria for seronegative rheumatoid arthritis (*ref. 19*).

'Large joints' refers to shoulders, elbows, hips, knees, ankles and spine.
'Small joints' refers to the metacarpophalangeal, interphalangeal, metatarsophalangeal and wrist joints.

Low ACR-scores were assigned to involvement of : 'one large joint' (1), '2-10 large joints' (2) and '1-3 small joints (with or without involvement of large joints)' (3).
High ACR scores were assigned to involvement of : '4-10 small joints (with or without involvement of large joints)' (4), or 'involvement of more than 10 joints (involving at least one small joint)' (5).

Table 3. Risk factors for chikungunya virus-associated long-term polyarthralgia (n=160)

Risk factors	Pain duration: < 6 weeks		> 6 weeks						> 1 year					
	n= 90 (100 %)		n=70 (100 %)		OR [95%-CI]				n=42 (100%)		OR [95%-CI]			
					Univariate		*Multivariate*				*Univariate*		*Multivariate*	
Age, yrs [IQR]	50	[35-63]	50	[37-56]					49	[37-54]				
≤ 40	25	(27.8)	20	(4.3)	-				13	(31.0)	-			
> 40	65	(71.4)	50	(24.3)	1.0	[0.5-2.0]			29	(69.0)	1.1	[0.5-2.5]		
Female gender	52	(57.8)	57	(81.4)	3.2	[1.6-6.9]	3.4	[1.6-8.0]	36	(85.7)	4.4	[1.8-12.5]	5.9	[2.1-19.6]
Obesity	6	(6.6)	17	(24.3)	4.5	[1.8-13.1]	4.8	[1.8-14.7]	10	(23.8)	4.4	[1.5-13.8]		
Diabetes	8	(8.8)	4	(5.7)	0.6	[0.2-2.1]			2	(4.8)	0.5	[0.1-2.2]		
Rheumatol. condition	3	(3.3)	1	(1.4)	0.4	[0.0-3.4]			1	(2.4)	0.7	[0.0-5.7]		
Osteoporosis	5	(5.5)	0						0					
Osteo-arthritis	5	(5.5)	4	(5.7)	1.0	[0.3-4.1]			3	(7.1)	1.3	[0.3-5.7]		
Sleep *	25	(27.4)	32	(45.7)	2.2	[1.1-4.3]			22	(52.4)	2.9	[1.3-6.2]		
Concentration *	12	(13.2)	26	(37.1)	3.8	[1.8-8.6]	4.7	[2.1-11.1]	17	(40.5)	4.4	[1.9-10.7]		
Redness	17	(18.7)	17	(24.3)	1.4	[0.7-3.0]			9	(21.4)	1.2	[0.5-2.9]		
Edema	26	(28.9)	36	(51.4)	2.5	[1.3-4.9]			25	(59.5)	3.6	[1.7-7.9]		
Stiffness	58	(64.4)	56	(80.0)	2.2	[1.1-4.6]			34	(81.0)	2.3	[1.0-5.9]		
Finger	39	(43.3)	44	(62.9)	2.2	[1.2-4.1]			31	(73.8)	3.7	[1.7-8.5]		
High ACR-score	37	(41.1)	45	(64.3)	2.6	[1.4-5.0]	2.9	[1.4-6.1]	33	(78.6)	5.3	[2.3-12.9]	7.4	[2.7-23.3]
RT-PCR positive > 7 DPSO¶	4/20	(20.0)	11/22	(50.0)	4.0	[1.1-17.6]			8/13	(61.5)	6.4	[1.4-34.1]		

To control for confounding we performed Stepwise Logistic Regression, using backward elimination for any variable (including age) whose univariate test p-value < 0.10.

** Sleep and concentration disorder were identified as risk factors for arthralgia; however, in our study it was not possible to distinguish whether these were predictors or consequences.*

DPSO denotes days post symptom onset.

¶ The odds ratios were calculated for 42 of 160 patients, whose sera were collected after 7 DPSO.

Discussion

The study of confirmed CHIKV cases during the 2014-2015 outbreak in Aruba enabled us to assess the relative contribution of serologic and molecular assays to the diagnosis of acute CHIKV, the clinical presentation and risk factors for pCHIK-CPA. CHIKV infection was confirmed in 269 out of 498 (54%) symptomatic patients, who had been referred by their family physicians for laboratory confirmation of suspected acute infection. This high frequency of confirmed CHIKV infection probably reflected the high prevalence of CHIKV during the outbreak and the rather good selection of candidates for testing based on clinical characteristics. However, we highlight that the absence of CHIKV nucleic acid detection assays in diagnostic facilities on the island led to substantial underdiagnosis in the acute phase of infection. This suggests that the incidence rates of suspected and confirmed CHIKV cases in Aruba as reported to PAHO (443 and 863 per 100.000, respectively), probably underestimated the true magnitude of the outbreak (20,21).

After a first introduction of CHIKV in immunologically naïve populations, reported attack rates vary widely in seroprevalence studies (10 to 75%) (7). These high attack rates have been attributed to high levels of viremia following CHIKV infection, to short incubation periods (both intrinsic, *i.e.* in the human host, and extrinsic, *i.e.* in the mosquito vector), and to the presence of competent vector populations (22).

By our study design, we could not identify asymptomatic infections or symptomatic individuals who did not seek consultation. In contrast to Flavivirus infections, asymptomatic CHIKV infections appear less frequent. Ranges from 3-28% have been reported from outbreaks involving ECSA genotype CHIKV (23–26). However, in the study by Gay *et al.* from Saint Martin 39.0% of CHIKV infections were asymptomatic (27). Not all symptomatic CHIKV infected patients seek medical attention. In Puerto Rico, laboratory evidence of recent CHIKV infection was found in 28% household contacts of confirmed CHIKV cases who did not consult a physician, even though 84% reported compatible symptoms (26).

In the Caribbean, early estimates of CHIKV incidence ranged from 52 to 115 cases per 1,000 after the emergence of an Asian genotype CHIKV in 2013 (28). Seroprevalence data obtained seven months after the outbreak in Saint Martin suggested that 16.9% of the population had been infected (27). Assuming an attack rate of 10% in Aruba's population of 100,000, we estimate that our cohort represents approximately 2.5% of the total number of CHIKV cases during the 2014-2015 outbreak.

Diagnostics

Using IFA, CHIKV-specific IgM was detected in the sera of 210 of 269 confirmed acute cases in our cohort. Ninety-seven percent of samples were collected at 3 DPSO or later. Although plaque reduction neutralization tests to increase specificity were not performed, false positive IFA results are unlikely, because alphavirus outbreaks had not been recorded previously on Aruba, and because of the high analytical specificity of the IFA (29). The accuracy of CHIKV diagnostic tests is obviously correlated to the time kinetics of viremia and specific antibody responses in human CHIKV infection.

CHIKV-specific IgM

The sensitivity of the IgM in the IFA only approximated 80% or higher after 6 DPSO (Fig 3). Post-hoc CHIKV-specific RT-PCR was positive in the sera of 164 of 269 (61%) cases. In the acute phase, the diagnostic yield additional to IgM detection alone was 28.1%. Other studies reported detection of IgM (and IgG) in 100% of cases from 5 or 6 DPSO onward (16, 30).

Variation in epitope recognition between the epidemic strains might explain differences in sensitivity of tests that detect IgM. This was observed in an evaluation of commercial serological assays (including the Euroimmun IFA) between the two subsequent ECSA genotype CHIKV outbreaks in Singapore, one of which involved the E1-A226V mutation (31). Variation between the Asian genotype CHIKV in Aruba and the ECSA genotype CHIKV that is used as an antigen substrate in the IFA, may have affected IgM detection in our cohort.

Another hypothesis to explain the reduced IgM detection rates in our cohort at 7 DPSO could be slower induction of CHIKV-specific antibody responses following infection with Asian-Caribbean genotype CHIKV compared to epidemic strains in previous reports because of differences in immunogenicity (32).

CHIKV-specific IgG

We detected CHIKV-specific IgG antibodies from 3 DPSO onwards. Detection of IgG this early is unexpected. Published data suggest that IgG does not appear until 6 DPSO, usually 1-2 days after IgM (13,16,30). The time-kinetics of the CHIKV-specific IgG response in relation to IgM and RNA results seems compatible with acute infection (Fig 3). Isolated IgG-positive results (*i.e.* in sera with negative RT-PCR and IgM results) were a rare finding in our cohort, and occurred only at the height of the epidemic or later. We therefore believe that all positive antibody detection results indicate recent CHIKV infection during the outbreak we describe here.

CHIKV-specific RT-PCR

The highest proportion of viremic cases (88%) was found at 4 DPSO. CHIKV RNA was detected in spite of the presence of IgM and IgG in up to 45% and 30% of sera, respectively. This is an unexpected finding, for experimental observations had suggested that CHIKV-specific antibodies had strong neutralizing activity in vitro and even therapeutic potential against CHIKV infection in mice (33). Jain *et al.* postulated that appearance of CHIKV-specific IgG is required to neutralize the virus (34). In accordance, previous observations of CHIKV infection in humans coupled the development of a CHIKV-specific antibody response to a rapid decrease in viremia (13,30,31). In studies from CHIKV outbreaks that employed both types of tests throughout the acute phase of infection, combined positive results for both RT-PCR and antibody detection based assays do not appear frequent, *i.e.* 1-12%. (35–38). However, our findings are in line with an analysis at California's National Reference Laboratory, that detected CHIKV-RNA in 34% and 36% of 376 IgM and IgG positive sera, respectively (39). Sequencing was not performed in that study, but Asian genotype was assumed since samples were obtained from travellers returning from the Caribbean in 2014.

Interestingly, 40 to 50% of patients in our cohort were viremic beyond 7 DPSO. This contrasts with frequent reports of CHIKV-RNA declining to undetectable levels within a week (16,35,40–43). One small study from Singapore found viremia to persist for more than a week in 30% of cases (44). In a large cohort from India, viral RNA was detected in patients who were recruited as late as 12 DPSO (34). In a Thai study, quantitative RT-PCR detected viremia up to 6 DPSO, but when using a second, nested RT-PCR protocol, CHIKV-RNA was found up to 9 DPSO in all of 45 patients (30). Detection of viral RNA depends on the sensitivity of the assays employed for nucleic acid detection. Variation in CHIKV genotypes or epidemic strains might account for observed differences in viral kinetics between outbreaks.

Clinical presentation

Arthralgia, fever, myalgia, headache and skin rash were the most frequent acute phase symptoms reported by CHIKV infected patients in our telephone survey (Table 1). Many patients complained of dizziness, but no other neurological symptoms were recorded. Sleeping and concentration disorders were recorded frequently, but it is impossible to determine whether they were caused by the infection or resulted from illness and pain. The clinical presentation in this study is largely consistent with reports from recent outbreaks, including those caused by strains derived from the Asian CHIKV lineage that affected territories in the Caribbean and South and Central America (43,45–48). A notable difference is that as many as 20% of patients in our adult study population experienced cough, sore throat or dyspnoea. Respiratory symptoms associated with CHIKV infection have not been reported consistently, but occurred in 23-30% of cases in epidemics in the Philippines, Trinidad and Tobago, and the Dominican Republic (36,38,49). Critical illness and death due to pneumonia and respiratory failure were reported in ECSA genotype CHIKV-infected patients during the outbreak at Réunion (50). It remains unclear whether these cases were caused directly by CHIKV, or by co-infection with respiratory pathogens to which CHIKV infection may predispose (51).

In line with published data from the 2014 Asian lineage outbreak in the Caribbean, no CHIKV-associated mortality was recorded in our series. Mortality resulting from CHIKV infection is thought to be rare and restricted to patients with atypical presentations or comorbidities, although some authors reported case-fatality ratios of up to 5% (6,52,53).

Arthralgia

The dominant clinical symptom in confirmed CHIKV infection was joint pain. Inflammatory characteristics such as morning stiffness, edema and redness were commonly reported. As a rule, multiple joints were symmetrically affected (resulting in a high ACR-score), with the fingers, knees, ankles and feet most commonly involved. pCHIK-CPA occurred frequently.

Previous reports vary greatly in mode of assessment, description, follow-up and classification of CHIKV-associated joint pains (10). After clinical evaluation, pCHIK-CPA has been diagnosed as arthritis, spondyloarthritis, tenosynovitis, fibromyalgia and arthralgia syndrome among others (54–57). The pathogenesis of CHIKV-associated arthropathy remains incompletely understood. The pattern of leucocyte infiltration, cytokine production, and complement activation in CHIKV-infected joints is similar to that found in (seronegative) rheumatoid arthritis (22,58). Clinically, inflammatory joint destruction by synovitis, erosions and joint space narrowing has been well documented (57,59).

The estimates of long-term arthralgia occurrence after CHIKV vary widely (10,60). Studies in mice suggest that the Asian CHIKV lineage from the Caribbean induces less severe joint pathology than the ECSA genotype from Réunion (61). Other sources of variation in disease severity between outbreaks may be differences in host response. Variation is certainly affected by differences in study design, such as cohort size, patient selection, mode and timing of assessment of joint pains, and length of follow-up. Previously, arthralgia was found to persist in 57 to 67% of CHIKV infected patients following the ECSA genotype outbreaks in Réunion and Europe (55,62–65). These percentages, from studies with an approach and length of follow-up

comparable to ours, were supported by a prospective study of viremic patients from Réunion (56). However, well-designed studies from Sri Lanka and India involving ECSA genotype, found rheumatic pains to last considerably shorter (4.1 and 17.8% at 12 months) (66,67). In a study of 437 patients who did not have prior joint pains from India, rheumatologists diagnosed 57% with postviral polyarthralgia, 22% with postviral inflammatory polyarthritis and 1.4% with rheumatoid arthritis, 15 months following CHIKV infection (68).

Only a few estimates of chronic sequelae of CHIKV infection in the Americas have been published. In Colombia, Rodriguez-Morales et al. found that polyarthralgia persisted during a median follow-up of 21 weeks in 44.3% of 131 infected patients (69). In Suriname, 22.2% of 180 symptomatic and viremic CHIKV-patients in prospective follow-up, still reported arthralgia at 6 months (48).

Our data on pCHIK-CPA are consistent with these earlier data from Asian genotype CHIKV outbreaks in the Americas. They are also consistent with a meta-analysis that conservatively estimated the proportion of patients with chronic inflammatory rheumatism following CHIKV-infection at 25%, and the percentage developing arthritis at 14% (60).

Predictors of CHIKV-associated long-term arthralgia

Analysing our clinical data, three independent risk factors for arthralgia lasting longer than 6 weeks held significance in multivariate analysis: female gender, obesity and a high ACR-score. Female gender and a high ACR-score also predicted pCHIK-CPA longer than one year. Age and co-morbidities other than obesity, were not associated with long-term arthralgia. Sleep and concentration disorders were correlated with persisting joint pains, but our study design did not enable us to distinguish cause from consequence. In addition, prolonged detection of CHIKV-RNA in serum at 8 to 10 DPSO was identified as a predictor for pCHIK-CPA at 6 weeks and at 1 year.

In our study, the number of female patients was twice that of men in both confirmed CHIKV and non-CHIKV cases. We therefore postulate that the female-to-male ratio in our study reflects gender differences in health seeking behaviour, rather than genuine differences in exposure to CHIKV or frequency of symptomatic infection. The seroprevalence rates from an outbreak that involved the same CHIKV genotype Saint Martin in 2014, also showed similar rates of infection between men and women (27). Differential susceptibility to CHIKV infection has been reported for either sex in studies from different outbreaks (70). Female gender has been mentioned as a risk factor for long-term joint pains in many CHIKV outbreaks (48,55,71–73). However, it has not been identified consistently, and pathophysiological evidence for a gender difference is lacking (64,74,75).

Age above 30 to 50 years was a risk factor in many reports (48,56,63,71,74–76). In line with studies by Larrieu *et al.* and Win *et al*., that involved ECSA genotype, age did not emerge as a risk factor in our univariate analysis, which excluded participants younger than 18 years (64,73).

Obesity, a major public health problem in Aruba, predicted pCHIK-CPA. For joint pains longer than 6 weeks duration, this self-reported finding was an independent predictor of commonly associated co-morbidities such as diabetes or degenerative joint disease, which were identified as risk factors in other studies (56,63). Obesity was also identified as an independent risk factor for CHIKV-induced arthritis in a large study from India (77). The authors suggested that obesity may lead to a chronic inflammatory state, that is aggravated by CHIKV infection. This hypothesis merits further study, especially since others found a trend of persisting arthralgia in patients with increased body mass index, even though it did not reach statistical significance (64,75).

In contrast with previous findings, long-term arthralgia was not associated with pre-existing rheumatological disease or other comorbidities (55,56,63). The relatively low prevalence of comorbid conditions in our cohort precludes generalization of these results.

Signs of inflammatory arthropathy and involvement of the fingers predicted long-term arthralgia in univariate, but not multivariate analysis. Instead, a high ACR-score in the acute phase was a significant risk factor for pCHIK-CPA. We therefore concur with the French guidelines for the management of chikungunya, to make an accurate assessment of the number of joints involved in the initial evaluation of CHIKV-infected patients (78).

Presence of CHIKV-RNA in serum after the first week was a strong predictor of pCHIK-CPA. This may point towards high viral loads at the time of symptom onset, which have been associated with prolonged joint pains in ECSA genotype infection (34,79). However, another study failed to demonstrate an association between peak viral load or duration of viremia with persistent arthralgia at 6 weeks (73). Following infection in humans, CHIKV rapidly mutates during the acute phase. Thiberville *et al.* observed that the increasing intra-host genetic diversity, with resulting higher viral amino-acid complexity in the acute phase, correlated with increased frequency of pCHIK-CPA at 300 DPSO (40). The formation of intra-host quasi-species may help CHIKV to evade a mounting adaptive immune response, and could explain prolonged persistence of CHIKV-RNA in spite of detectable CHIKV-specific antibodies. The advent of affordable next generation sequencing instruments should enable us to investigate this hypothesis in the near future.

Finally, pCHIK-CPA was associated with extended periods of sick leave. Although mortality and hospitalization rates appear lower than those reported for the ECSA genotype outbreaks in Asia, the high attack rates of CHIKV in the Americas are associated with considerable morbidity. The increase in consultations and absenteeism we reported here, seem a crude measure of the direct and indirect health costs associated with CHIKV outbreaks.

Limitations

Our retrospective study was subject to a number of limitations. Recall bias could not be excluded. The time lapse between the survey and disease onset might have affected acute phase reporting in particular. The structured questionnaire used multiple choice questions, which restricted the amount of detail we recorded. Further, description of inflammatory characteristics and arthralgia classification was based on self-reporting, which was reported to overestimate the number of joints involved and the presence of signs compared to assessment by a qualified physician (62). A prospective study has been planned to assess structural joint damage in patients with persisting pCHIK-CPA in Aruba. Public awareness of lack of specific antiviral treatment probably prevented many symptomatic patients from seeking medical consultation, and may have introduced selection bias in our cohort. As with other outbreak reports, the unique interaction between infecting CHIKV strain and host population limits generalizability of our study results.

Conclusions

We report on the first outbreak of an Asian lineage of CHIKV in Aruba. More than one fifth of cases would not have been detected if RT-PCR testing was omitted. We emphasize the need for a combined molecular and serological approach to diagnose CHIKV in a single sample during the acute phase. Rapid diagnostic tests, that meet the ASSURED criteria (affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free and delivered on location) are urgently needed (80). CHIKV-antigen detection tests are being developed, but unsatisfactory diagnostic performance across different genotypes does not permit commercialization yet (81,82).

Our survey of the clinical presentation of CHIKV infection in an adult population indicated that arthralgia, fever and skin rash were the dominant acute phase symptoms. Arthralgia was characterised by signs of inflammation in up to 71% of those affected. Forty-four percent of

cases had pCHIK-CPA for longer than 6 weeks, and 26% for at least one year. In Aruba, a study has been planned to classify rheumatic patterns and to assess structural joint damage in patients with persisting pCHIK-CPA.

We identified female gender, obesity, a high ACR-score and prolonged detection of CHIKV-RNA in serum (longer than 1 week after symptom onset) as predictors for pCHIK-CPA. These risk factors should be validated in prospective studies, so they could contribute to the timely identification of CHIKV-infected patients who are likely to benefit from future therapeutic interventions, such as disease-modifying anti-rheumatic drugs or antiviral compounds (78,83–85).

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Chapter 2a

A rapid test for chikungunya virus ?

Diagnostic accuracy of a rapid E1-antigen test for chikungunya virus infection in a reference setting

Ralph Huits [1], Tamaki Okabayashi [3], Lieselotte Cnops [1], Barbara Barbé [1], Riemsdijk Van Den Berg [4], Koen Bartholomeeusen [2], Kevin K. Ariën [2], Jan Jacobs [1,5], Emmanuel Bottieau [1], Emi E. Nakayama [3], Tatsuo Shioda [3], Marjan Van Esbroeck [1]

[1] Department of Clinical Sciences, Institute of Tropical Medicine, Antwerpen, Belgium;
[2] Department of Biomedical Sciences, Institute of Tropical Medicine, Antwerpen, Belgium;
[3] Mahidol-Osaka Center for Infectious Diseases (MOCID), Research Institute for Microbial Diseases, Osaka University, Osaka, Japan;
[4] Horacio Oduber Hospital/ Landslaboratorium Aruba, Oranjestad, Aruba;
[5] Department of Microbiology and Immunology, University of Leuven, Leuven, Belgium

Keywords: chikungunya virus, diagnostics, rapid antigen test

Clinical Microbiology and Infection. 2018 Jan;24(1):78-81.

Abstract

Objectives

Rapid diagnostic tests targeting virus-specific antigen could significantly enhance the diagnostic capacity for chikungunya virus infections. We evaluated the accuracy of an immunochromatographic antigen test for the diagnosis of chikungunya in a reference laboratory for arboviruses.

Methods

An immunochromatographic rapid test that uses mouse monoclonal antibodies as a tracer against the E1-envelope protein of chikungunya (ARKRAY, Inc. Kyoto, Japan) was evaluated. Sensitivity was tested in sera from travellers with RT-PCR confirmed chikungunya virus infection (Eastern/Central/Southern African (ECSA) genotype) (n=9) and from patients diagnosed during the 2014-2015 chikungunya outbreak on Aruba (Asian genotype, n=30). Samples from patients with other febrile and non-febrile illnesses (n=26), sera spiked with *Flavivirus* and *Alphavirus* reference strains (n=13, including non-spiked serum) and samples containing other selected pathogens (n=20) were used to test specificity of the E1-antigen test.

Results

Sensitivity of the E1-antigen test was 8/9 (88.9%, 95%-confidence interval (CI)[56.5 – 98.0]) for the ECSA genotype, but only 10/30 (33.3%, 95%-CI [19.2 - 51.2]) for the Asian genotype. Overall diagnostic specificity was 49/59 (83.1%, 95%-CI [71.5 - 90.5]).

Conclusions

The E1-antigen test we evaluated had fair diagnostic sensitivity for ECSA genotype chikungunya, but low sensitivity for Asian genotype, and poor overall specificity. Antibodies that react across genotypes will be required for further development of a rapid test for chikungunya. Performance of new tests should be evaluated against different chikungunya genotypes.

Introduction

Chikungunya fever is characterized by fever, incapacitating arthralgia and rash (1), and is frequently followed by long-lasting arthralgia (2). It is caused by infection with chikungunya virus (CHIKV), a single-stranded positive-sense RNA virus of the family *Togaviridae*. Three genotypes have been described: West African, Eastern/Central/Southern African (ECSA) and Asian (3). ECSA and Asian genotype have caused large-scale outbreaks globally. In 2013, an Asian lineage of CHIKV was introduced into the Caribbean (4).

Diagnosis of CHIKV infection is based on detection of viral RNA by Reverse Transcription- Polymerase Chain Reaction (RT-PCR) in the viraemic phase, or by detection of anti-CHIKV IgM and IgG antibodies by enzyme-linked immunosorbent assay (ELISA) or Indirect Immune Fluorescence Testing (IIFT) (5). For clinical and epidemic reasons, a point-of-care test would be an asset (6). Okabayashi *et al.* developed an immunochromatographic (IC) assay with mouse monoclonal antibodies (MAbs) targeting the E1 envelope proteins of CHIKV: sensitivity and specificity were 89.4% and 94.4% respectively, but the Asian genotype was underrepresented in their study (7). We evaluated the diagnostic accuracy of a CHIKV E1-antigen test that uses the same antibodies, on a panel comprising the Asian genotype.

Methods

The CHIKV E1-antigen test (E1-Ag test, manufactured by ARKRAY, Inc. Kyoto, Japan) is a nitrocellulose membrane strip used as a dipstick. The sample pad contains CK119 MAb conjugated to colloidal gold; the test and control lines use CK47 MAb respectively anti-mouse IgG antibody (Immuno Probe Co. Ltd, Saitama, Japan). The MAbs CK47 (IgG2a subtype) and CK119 (IgG1) were generated as described previously [7-8].

According to the manufacturer's instructions, 30 µl of buffer and 30 µl serum were mixed in a tube, in which the dipstick was placed upright. After 15 minutes, the dipstick was removed from the tube, placed

horizontally and read by two readers blinded to each other; final results were based on consensus. Test line intensity was scored as 'faint' (hardly visible) and 'weak', 'medium' or 'strong' if intensity was weaker, equal or stronger compared to the control line. Incomplete background absence of the strip and absence of the control line (invalid tests) were recorded. Readings were repeated at 30 minutes (*i.e.* after the recommended reading delay). All sample codes were concealed from the investigators until all results were recorded.

Diagnostic sensitivity was evaluated against both ECSA and Asian genotype CHIKV using CHIKV-specific RT-PCR as reference method (8). (Table 1). The ECSA genotype panel (n=9) consisted of CHIKV positive serum samples from travellers diagnosed at the Institute of Tropical Medicine (ITM) between 2006 and 2014 ((9). ECSA genotype was confirmed in four samples; three sequences showed 226V (ECSA, Indian Ocean Lineage), the remaining showed 226A in the E1 coding region. ECSA genotype for the other samples was assumed because of the prevailing strains at the place and time of epidemic. Asian genotype CHIKV serum samples (n=30) were obtained from patients during the 2014-15 CHIKV epidemic in Aruba, an island in the Dutch Caribbean. All Asian genotype samples had been collected within 10 days post symptom onset (pso) and were RT-PCR positive. For antibody detection, ITM and Landslaboratorium Aruba used the anti-Chikungunya Virus IgM/ IgG IIFT (Euroimmun, Lübeck, Germany).

14/30 tested anti-CHIKV IgM positive and all tested anti-CHIKV IgG negative. Samples had been stored at -80°C until shipment to ITM and again after RT-PCR until evaluation of the E1-Ag test. CHIKV was isolated from 7 samples with Ct-values below 30 by inoculation onto Vero cells. The E1-region (position 9994-11310) of these isolates was genotyped (Sanger sequencing method); all clustered with the Asian-Caribbean strain. The A226V mutation was absent.

Diagnostic specificity was assessed in a panel of sera from patients with other febrile illnesses, collected during the 2014-15 CHIKV epidemic in Aruba (endemic control panel, n=26), archived sera of travellers at ITM

(selected pathogens panel, n=20), and a reference strain panel spiked in sera (Table 2). Six samples from the endemic control panel were positive in the RT-PCR for dengue virus (DENV). The spiked sera of the reference panel contained 1 µl of viral culture supernatant per 100 µl (Ct-values 12-18); in addition a non-spiked sample was added.

Results

The E1-Ag test showed test lines in 8/9 samples of the ECSA panel, corresponding to a sensitivity of 88.9% (95%-CI [56.5 – 98.0]) (Table 1). The false-negative result occurred in a sample from a traveller to Mauritius, obtained on day 3 pso. Among the Asian genotype panel, sensitivity was 33.3% (10/30 samples (95%-CI [19.2 - 51.2]). Only 2/7 culture positive CHIKV samples tested positive. Appearance of test lines was not related to the number of days pso. Line intensities on true positive tests were scored as medium 2/18 (11.1%), weak 6/18 (33.3%) and faint 10/18 (55.6%) (Fig. 1).

Overall diagnostic specificity was 49/59 (83.1%, 95%-CI [71.5 - 90.5]). In the endemic control panel (Table 3) test lines were visible in 6/26 samples, one of which was DENV RT-PCR positive (Ct-value 38, DENV serotype 1); test line intensities were faint (n=4) and weak (n=1). The corresponding diagnostic specificity was 20/26 (76.9%, 95%-CI [57.9 – 89.0]). In the selected pathogens panel, specificity was 75.0%, (95%-CI [53.1 – 88.8]); false-positive samples included those with *Trypanosoma brucei rhodesiense antibodies*, *Plasmodium falciparum*, *Plasmodium malariae*, *Plasmodium vivax* and hypergammaglobulinaemia (one sample each, weak and faint test lines). Among the reference panel (specificity 91.7%, 95%-CI [64.6 – 98.5]), the Sindbis virus sample showed a faint test line.

No invalid results were recorded. The majority of tests (78/97, 79.6%) displayed incomplete background clearance. Inter-observer agreements were fair (92.9%, Kappa value: 0.83 (95% CI [0.71 - 0.95]). Delayed reading at 30 minutes increased the diagnostic sensitivity for the Asian genotype to 46.7% (14/30 samples, 95%-CI [30.2 – 63.9]), but specificity decreased to 74.6% (95%-CI [62.2 – 83.9]).

Discussion

When tested in a reference setting, the diagnostic sensitivity of the E1-Ag test (ARKRAY) for the ECSA genotype was good (88.9%) and similar to that reported for a product that used the same MAbs (7). However, the sensitivity for the Asian genotype was low (33.3%). Repeated freeze-thaw cycles are not a likely explanation for this lower sensitivity nor are the delays pso (IgM seroconversion rates of the Asian and ECSA panels were similar (14/30 and 4/9, respectively)). Although the presence of antibody-antigen complexes (such as in DENV infections) may influence the reactivity in the E1-Ag test (10), we believe that the low sensitivity for the Asian panel is most probably explained by the low affinity of the MAbs. Indeed, the amino acid substitution D350E in the 6K-E1 protein of CHIKV (corresponding to position 284 in the E1 protein-coding region) is absent in the Asian genotype, and the E-to-G substitution at this position has been shown to reduce the CK47-mediated suppression of virus growth (11). Characterization of CK47 and CK 119 MAb binding sites is currently the subject of further research. The overall diagnostic specificity was moderate, particularly among the CHIKV-negative endemic panel (76.9%); further research must be done to clarify the reasons and improve specificity.

It is clear that the poor sensitivity and moderate specificity preclude the use of the present version of the E1-Ag test. In addition, operational characteristics (background clearing, stability of test lines, line intensities) need to be improved. For point-of-care testing, a cassette form will be more user-friendly and safe compared to the present dipstick format.

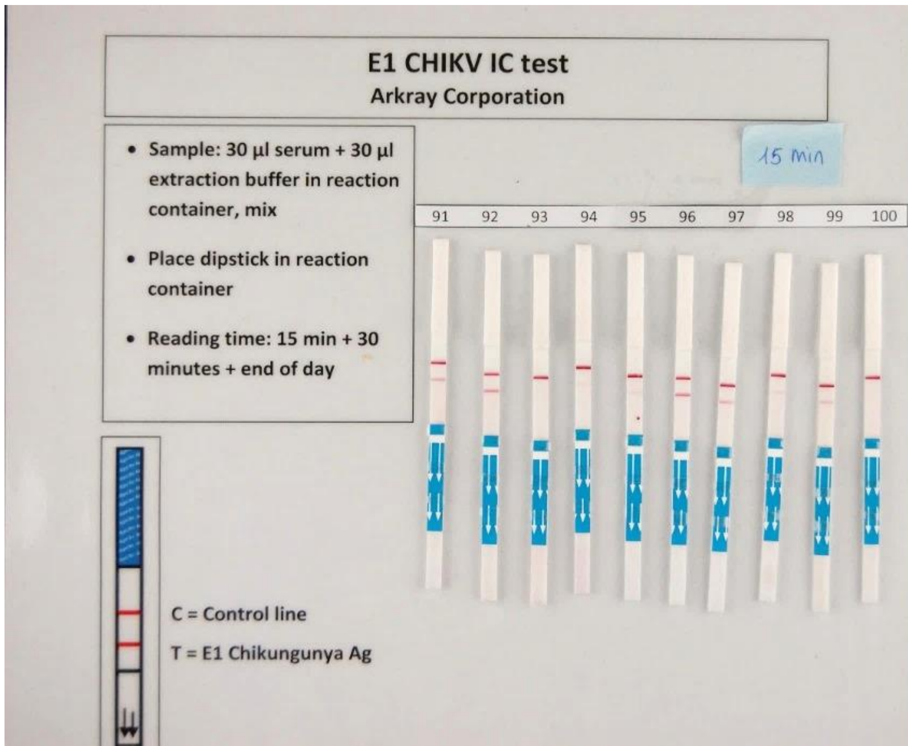
Table 1. Chikungunya E1-antigen test results in ECSA genotype panel

sample number	Country	DPSO #	anti-CHIKV IgM°	anti-CHIKV IgG°	CHIKV culture	CHIKV RT-PCR°° Ct-value	E1-antigen test result*
1	Mauritius	2	-	-	ND	34	W
2	Reunion	2	-	-	ND	18	M
3	Mauritius	3	+	+	ND	37	N
4	Mauritius	3	+	+	ND	35	F
5	Mauritius	3	ND	ND	ND	34	W
6	India	2	ND	-	ND	20	M
7	Thailand	4	+	+	ND	35	W
8	Thailand	2	-	ND	ND	29	W
9	French Polynesia	1	-	-	ND	19	W

(legend

 # DPSO denotes 'Days post symptom onset'
° anti-Chikungunya Virus IgM/ IgG IIFT assay (Euroimmun, Lübeck, Germany)
°° CHIKV-specific RT-PCR (4), Ct-value: Cycle threshold value
** E1-antigen test results F (faint or hardly visible), W (weak), M (medium) or S (strong) if test line intensity weaker, equal or stronger compared to the control line, were considered positive. E1-antigen test results N (no test line visible), was considered negative.*
ND: not done
Undeterm.: undetermined

Figure 1. Test line intensities



Test line intensity was scored as:
'faint' - hardly visible, as in test strips no. 94, 95 and 98;
'weak' - as in test strips no.91, 97 and 99;
'medium' - as in test strips no. 92 and 96; or
'strong', if intensity was weaker, equal, or stronger compared with the control line.

Table 2. Chikungunya E1-antigen test results in Asian genotype panel

sample number	Country	DPSO #	anti-CHIKV IgM°	anti-CHIKV IgG°	CHIKV culture	CHIKV RT-PCR°° Ct-value	E1-antigen test result*
10	Aruba	5	+	-	ND	38	W
11	Aruba	6	-	-	+	22	N
12	Aruba	5	+	-	ND	36	F
13	Aruba	5	+	-	ND	36	N
14	Aruba	4	+	-	ND	34	N
15	Aruba	6	-	-	-	29	F
16	Aruba	4	+	-	ND	36	F
17	Aruba	4	Undeterm.	-	+	29	N
18	Aruba	4	-	-	+	25	F
19	Aruba	5	+	-	ND	40	N
20	Aruba	3	-	-	+	17	N
21	Aruba	3	-	-	+	26	F
22	Aruba	4	-	-	ND	36	N
23	Aruba	5	-	-	ND	33	N
24	Aruba	4	-	-	ND	35	N
25	Aruba	5	-	-	ND	37	F
26	Aruba	5	Undeterm.	-	ND	34	N
27	Aruba	3	-	-	+	23	F
28	Aruba	5	-	-	+	20	N
29	Aruba	5	Undeterm.	-	ND	34	N
30	Aruba	5	+	-	ND	35	N
31	Aruba	9	-	-	ND	33	N
32	Aruba	10	-	-	ND	34	F
33	Aruba	5	-	-	ND	36	N
34	Aruba	4	-	-	ND	37	N
35	Aruba	0	+	-	ND	33	N
36	Aruba	5	-	-	-	31	F
37	Aruba	7	+	-	ND	38	N
38	Aruba	9	+	-	ND	37	N
39	Aruba	6	+	-	ND	37	N

Table 3. Chikungunya E1-antigen test results in chikungunya, other febrile conditions, selected pathogens and spiked sera panels

Test panel	CHIKV RT-PCR positive (n)	E1-Ag positive	E1-Ag negative	Sensitivity (%) [95% - CI]#	Specificity (%) [95% - CI]#
chikungunya, ECSA genotype, n=9	9	8	1	88.9% [56.5- 98.0]	
chikungunya, Asian genotype, n=30	30	10	20	33.3% [19.2- 51.2]	
endemic controls, n=26	ND	6	20	-	76.9% [57.9 – 89.0]
Selected pathogens°, n=20	ND	5	15	-	75.0% [53.1 – 88.8]
spiked sera°°, n=12	ND	1*	11	-	91.7% [64.6 – 98.5]

° *The selected pathogens panel included Plasmodium falciparum (n=2), P. vivax (n=2), P. ovale (n=2), P. malariae (n=2), Trypanosoma brucei rhodesiense (n=1), Schistosoma antibodies (n=2), Hepatitis C virus (n=2) and Epstein-Barr virus antibodies (n=2). Additionally, samples with hypergammaglobulinaemia (n=3) and increased Rheumatoid Factor concentration (n=2) were tested.*

°° *The reference panel (1 µl of viral culture supernatants per 100 µl) included Alphavirus (n=7): Ross River Virus, Sindbis Virus, Western equine encephalitis virus, Eastern equine encephalitis virus, Venezuelan equine encephalitis virus, Mayaro virus and o'nyong nyong virus; Flavivirus (n=5): West Nile Virus, Zika Virus, tick-borne encephalitis virus, yellow fever virus and Japanese encephalitis virus. Note: non-spiked serum (n=1) was added as a control and not included in the analysis.*

* *the E1-Ag test showed a visible test line in the Sindbis virus sample*

\# *95%- CI denotes 95% -confidence interval for binomial proportions (Wilson)*

ND: *not done*

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Chapter 2b

In Chapter 2a, we evaluated a prototype E1-antigen test for chikungunya virus (CHIKV). This test used mouse monoclonal antibodies (MAbs) CK47 (IgG2a subtype) and CK119 (IgG1).

The MAbs were generated using CHIKV strains from Sri Lanka (strain SL111314 isolated in 2009, ECSA genotype, GenBank accession no. AB455493.1) and from Thailand (strain CP10 isolated in 2010, ECSA genotype (Indian Ocean lineage), GenBank accession no. AB857817.1).

We postulated that the low sensitivity of the E1-antigen test for Asian genotype CHIKV was due to the low affinity of the MAbs for the E1 epitopes of Asian genotype CHIKV. This concept was studied further in CHIKV strains that did and did not possess the amino acid substitution D350E in the 6K-E1 protein.

The findings illustrate that 'Variation at position 350 in the Chikungunya virus 6K-E1 protein determines the sensitivity of detection in a rapid E1-antigen test', and were published by Aekkachai Tuekprakhon in a paper which I co-authored.

Chapter 2b contains the abstract, a figure and a table from this paper.

Variation at position 350 in the Chikungunya virus 6K-E1 protein determines the sensitivity of detection in a rapid E1-antigen test

Aekkachai Tuekprakhon [1,2], Emi E. Nakayama [2,3], Koen Bartholomeeusen [4], Orapim Puiprom [2], Tadahiro Sasaki [2], Ralph Huits [5], Natthanej Luplertlop [1], Nathamon Kosoltanapiwat [1], Pannamas Maneekan [6], Kevin K. Ariën [4,7], Tatsuo Shioda [2,3] & Pornsawan Leaungwutiwong [1]

[1] Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand.[2] Mahidol-Osaka Center for Infectious Diseases (MOCID), Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand.

[3] Research Institute for Microbial Diseases (RIMD), Osaka University, Osaka, Japan.

[4] Department of Biomedical Sciences, Institute of Tropical Medicine, Antwerp, Belgium.

[5] Department of Clinical Sciences, Institute of Tropical Medicine, Antwerp, Belgium.

[6] Department of Tropical Hygiene, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand.

[7] Department of Biomedical Sciences, University of Antwerp, Antwerp, Belgium.

[8] Mahidol-Osaka Center for Infectious Diseases (MOCID), Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand. shioda@biken.osaka-u.ac.jp.

[9] Research Institute for Microbial Diseases (RIMD), Osaka University, Osaka, Japan.

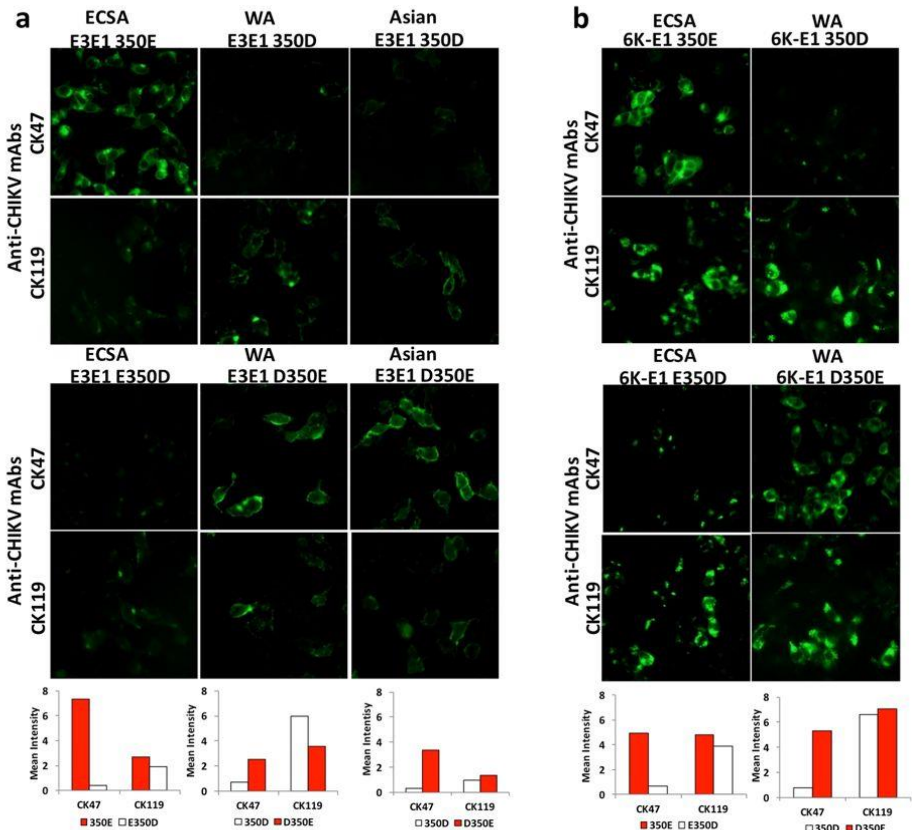
[10] Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand.

Scientific Reports. 2018 Jan 18;8(1):1094.

Abstract

Chikungunya virus (CHIKV), a mosquito-borne pathogen, consists of three genotypes: East/Central/South African (ECSA), West African (WA), and Asian. Although a current rapid immunochromatographic (IC) test detecting CHIKV E1-antigen showed high sensitivity to ECSA-genotype viruses, it showed poor performance against the Asian-genotype virus that is spreading in the American continents. To understand the basis for the low performance of this IC test against Asian-genotype virus, we re-examined the anti-CHIKV monoclonal antibodies (mAbs) used in the assay for their interaction with E1-antigen of the three CHIKV genotypes. We found that the reactivity of one mAb for Asian-genotype virus was lower than that for ECSA virus. Comparison of E1 amino acid sequences revealed that the ECSA virus used to generate these mAbs possesses glutamic acid (E) at position 350, in contrast to WA and Asian, which possess aspartic acid (D) at this position. Site-directed mutagenesis confirmed that the mutation altered mAb reactivity, since E-to-D substitution at position 350 in ECSA reduced recognition by the mAb, while D-to-E substitution at this position in Asian and WA increased affinity for the mAb. Taken together, these results indicate that residue 350 of the CHIKV 6K-E1 is a key element affecting the performance of this IC assay.

Figure 1. Amino acid at position 350 is a key element of CHIKV antigen: CK47 interaction.



Plasmids encoding CHIKV envelope protein from each of the three genotypes were transfected into HEK293T (Human Embryonic Kidney) cells and Indirect Immunofluorescence test was performed using CK47 and CK119; labeling was detected with Alexa Fluor 488-conjugated secondary antibody. Plasmids were constructed to encode the full-length CHIKV envelope protein (E3-E2–6K-E1) from the CP10 (ECSA), 37997 (WA), or CK12–686 (Asian) strain, or mutant envelope proteins from CP10 bearing E350D, 37997 bearing D350E, or CK12–686 bearing D350E (a). Plasmids were constructed to encode the short versions of the envelope protein (6K-E1) from the CP10 strain (ECSA) or 37997 (WA) strain bearing either the wild type or mutant residue at position 350 (b). Images are representative of results obtained from three independent experiments and were taken under 20X objective magnification. Red and white bars below photos are fluorescent signal levels of 350E and 350D versions of the indicated envelope proteins, respectively.

Table 1. Amino acid variation in CHIKV E1 proteins.

Country	Year	Strain	Accession No.	Genotype	Amino acid position																		
					34	72	98	145	162	211	225	226	269	276	284	296	304	321	343	344	379	404	420 *
					100	138	164	211	228	277	291	292	335	342	350	362	370	387	409	410	445	470	486 **
Tanzania	1952	S27	NC004162	Proto-type	L	N	A	T	I	K	A	A	M	M	D	L	P	A	E	I	E	A	V
Tanzania	1953	Ross Low psg	HM045811.1	ECSA	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Sri Lanka	2009	SL111314	AB455493.1	ECSA	.	.	.	.	.	.	.	.	V	.	E	.	.	.	.	.	.	.	.
Malaysia	2009	0901aTw	FJ807895.1	ECSA-IOL	.	.	.	.	.	.	.	V	V	.	E	.	.	.	.	.	.	.	.
Thailand	2010	CP10	AB857817.1	ECSA-IOL	.	.	.	.	.	.	.	V	V	.	E	.	.	.	.	.	.	.	.
Cambodia	2011	V1024314 KH11 PVH	JQ861258.1	ECSA-IOL	.	.	.	.	.	.	.	V	V	.	E	.	.	.	.	.	.	.	.
Brazil	2016	BR33	KX228391.1	ECSA	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.
Senegal	1983	37997	AY726732.1	WA	Q	S	.	A	V	.	.	.	V	I	.	V	.	T	D	V	A	T	I
Senegal	2005	HD 180760	HM045817.1	WA	Q	S	.	A	V	.	.	.	I	I	.	V	.	T	D	V	A	T	I
Thailand	1975	1455-75	HM045814.1	Asian	.	S	T	S	.	E	S	.	.	.	.	.	S	.	.	.	.	.	.
Indonesia	1983	JKT23574	HM045791.1	Asian	.	S	T	S	.	E	S	.	.	.	.	.	S	.	.	.	.	.	.
Philippines	2012	CK12-686	CWIH00000.1	Asian	.	S	T	A	.	E	S	.	.	.	.	.	S	.	.	.	.	.	.
Aruba***	2015	AUA-15801125	MF682981	Asian	.	S	T	A	.	E	S	.	.	.	.	.	S	.	.	.	.	.	.

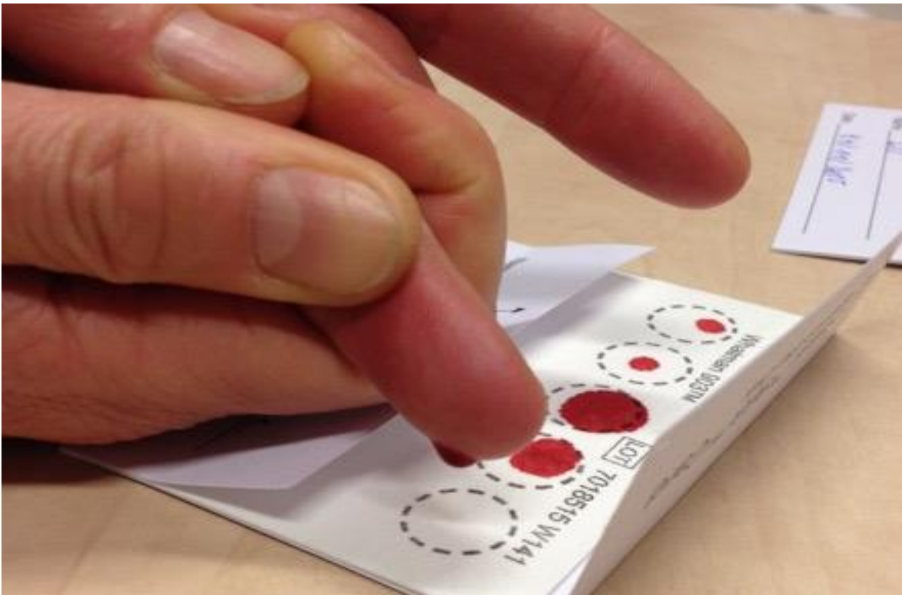
* *Amino acid position in E1 protein.*

** *Amino acid position in 6K-E1 protein.*

*** *Aruba isolates, ARUBA-15801136 (MF682982), 15801160 (MF682983),15801358 (MF682984), and 15801654 (MF682985) possess the same amino acid residues as ARUBA-15801125 at positions shown in this table*

Chapter 3

The risk of infection when travelling to 'Zika land'



Collection of capillary blood on a filter paper during travel, to facilitate post-hoc diagnosis (photograph by Ralph Huits)

Incidence of Zika virus infection in a prospective cohort of Belgian travellers to the Americas in 2016

Ralph Huits MD [1], Dorien Van Den Bossche MSc [1], Kaat Eggermont BSc [1], Erica Lotgering BSc [1], Anne-marie Feyens BSc [1], Idzi Potters BSc [1], Jan Jacobs MD PhD [1,2], Marjan Van Esbroeck MD [1], Lieselotte Cnops PhD [1#], Emmanuel Bottieau MD PhD [1#]

[1] Department of Clinical Sciences, Institute of Tropical Medicine, Antwerp, Belgium
[2] Department of Microbiology and Immunology, University of Leuven, Leuven, Belgium.

[#] These authors contributed equally to this manuscript

Key words: Zika virus; outbreak; Americas; incidence rate; prospective cohort study; Enzyme Linked Immunosorbent Assay; virus neutralization testing; real-time reverse transcription polymerase chain reaction; filter paper

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Abstract

Background

The incidence rate of Zika virus (ZIKV) infection in travellers from non-endemic areas to the Americas during the ZIKV outbreak in 2016 is unknown.

Methods

Belgian adults who planned to travel to South America, Central America, and the Caribbean were recruited prospectively to study the incidence and characteristics of ZIKV. Demographic data and sera were collected at baseline. Participants were trained to collect capillary blood on filter paper (BFP). When ill during travel, the participants completed a questionnaire and they sampled BFP for post-hoc analysis. All symptomatic participants were screened for ZIKV using ZIKV-specific RT-PCR on serum or urine, or BFP, and antibody detection assays (ELISA). Follow-up sera of asymptomatic travellers, obtained at least 20 days post travel, were tested by ZIKV ELISA only. All positive ELISA results were subject to confirmation by virus neutralization testing (VNT).

Results

Forty-nine participants completed follow-up: 38 women and 11 men, with a median age of 32 years (range 19–64 years). Travel destinations were countries in South America (n = 20), Central America (n = 24), and the Caribbean (n = 5). The total travel duration was 67.8 person-months. Illness was reported by 24 participants (49.0%). ZIKV infection was confirmed in nine cases, by RT-PCR (n = 5) and by VNT (n = 4). Only one of nine ZIKV cases (11.1%) was asymptomatic. The ZIKV incidence rate was 17.0% (95% confidence interval 7.8–32.2%) per month of travel.

Conclusions

The ZIKV incidence rate in adult travellers from non-endemic countries to the epidemic territories during the 2016 outbreak was high. Asymptomatic ZIKV infection was rare in this population.

Introduction

Following the epidemic spread of an Asian strain of Zika virus (ZIKV) in the New World, a causal association was recognized between ZIKV infection during pregnancy and adverse neonatal outcomes (1). Although primarily transmitted by *Aedes* spp. mosquitoes, evidence has emerged that ZIKV could also be transmitted through sexual intercourse (2,3). In addition, prolonged virus shedding has often been observed in the semen of infected men (4). Forty-eight countries in the Americas reported autochthonous vector-borne transmission of ZIKV in 2016-2017 (5). As many of these countries are popular travel destinations, travellers from non-endemic countries were particularly concerned about the risk of infection and of secondary sexual transmission upon return (6). Data collection by surveillance networks like GeoSentinel, has further indicated that travel-associated infection with ZIKV may drive its global spread (6).

However, incidence rates of ZIKV infection in travellers remain unknown because denominator data are unavailable (7). This prospective observational study was performed to assess the incidence of ZIKV infection in adults travelling to the Americas in 2016.

Methods

In February 2016, a prospective cohort study was started to determine the incidence and aetiology of febrile illness during travel in (sub-) tropical areas (protocol registered at ClinicalTrials.gov NCT02900066). As the start of recruitment coincided with the ZIKV outbreak in the Americas (8), it was aimed to determine the incidence and clinical characteristics of ZIKV infection in travellers to destinations in South America, Central America or the Caribbean from February to December 2016.

Belgian participants aged 18 years or older were actively recruited through travel organizations and educational institutions. Before travel, baseline demographic and clinical data were obtained during a

structured interview by a physician (RH) at the Institute of Tropical Medicine in Antwerp, Belgium. A baseline serum sample was stored. Participants were trained to draw blood from a finger-prick using an auto-retractable safety lancet, and to collect the capillary blood on filter paper (BFP) (Whatman 903 Filter Paper).

All participants were provided with a kit that contained the necessary study materials (see Supplementary Material). In the event of illness during travel, participants were requested to record data on symptoms, protective measures and exposure to health hazards, diagnostics, and case management abroad in a structured questionnaire. When symptoms matched the clinical criteria of the World Health Organization case definition for ZIKV infection, *i.e.* fever or rash (9), participants were instructed to collect BFP for laboratory diagnosis upon return from travel. Participants with diseased were further advised to seek medical attention during travel and they were offered consultations at the Institute of Tropical Medicine upon their return. All symptomatic and asymptomatic participants were also contacted by telephone for completion of data missing from the questionnaires and to request a follow-up serum sample to determine seroconversion rates.

For participants who were symptomatic during travel or at presentation to the Institute of Tropical Medicine, ZIKV-specific real-time reverse transcription PCR (RT-PCR) (RealStar Zika Virus RT-PCR Kit, Altona Diagnostics GmbH, Hamburg, Germany) was performed on BFP obtained during the acute phase (details provided in the Supplementary Material), or on serum or urine samples obtained within 7 or 14 days post symptom onset. Post-travel serum samples were screened for ZIKV-specific immunoglobulins (Ig) by a ZIKV IgG/IgM ELISA (Euroimmun, Lübeck, Germany). Serum samples from asymptomatic participants were obtained at least 20 days post travel. The baseline sera of all participants were assessed for the presence of antibodies against dengue virus (DENV) using the Dengue Virus IgM Capture DxSelect and Dengue Virus IgG DxSelect assays (Focus Diagnostics, Cypress, CA, USA), as indicators of previous DENV or related flavivirus infection. Samples for which positive or equivocal ELISA results were obtained

were further tested in a virus neutralization testing assay (VNT) using the 90% neutralizing antibody titre. In internal validation procedures, it was demonstrated that cross-reactivity with DENV antibodies for this VNT was limited to ZIKV VNT titres between 1:10 and 1:100. Symptomatic and asymptomatic participants with a positive RT-PCR or VNT result were defined as ZIKV cases, and participants with negative results were defined as non-ZIKV cases.

Primary ZIKV infection is believed to induce protective immunity (10,11). The time at risk was defined by subtraction of the travel time after symptom onset for cases from the total travel duration in endemic areas. As the time to symptom onset could not be ascertained for asymptomatic travellers, the median time to symptom onset was used for symptomatic participants in this study to estimate the time at risk for asymptomatic cases with VNT-confirmed ZIKV infection. The incidence rate of ZIKV infection in this cohort was then calculated by the formula: (number of cases) / ((travel duration of cases) – (travel duration of cases – time to symptom onset)) (see Supplementary Material). The 95% confidence interval for the incidence rate estimate was calculated using the Poisson distribution.

Results

A total of 55 participants were recruited, of whom 49 (89.1%) had a complete follow-up (38 women and 11 men). The median age was 32 years (range 19-64 years). Travel destinations were countries in South America (n = 20), Central America (n = 24), and the Caribbean (n = 5). The purpose of travel was tourism for 40 participants (81.6%), student internship for six participants (12.2%), and relief work for three participants (6.1%). The median duration of travel was 22 days (range 16-197 days), with a total duration of 2068 person-days (67.8 person-months). Twenty-four participants (49.0%) reported an illness, and nine cases of ZIKV infection were identified, including one asymptomatic case (Table 1).

The predominant symptoms in the ZIKV cases were rash, headache and diarrhoea. A skin rash was the only symptom that distinguished ZIKV infection from other illnesses (odds ratio 45, 95% CI 3.4–594). No neurological complications after ZIKV infection were observed.

Five ZIKV cases were diagnosed by RT-PCR: three on BFP collected during travel (cycle threshold (Ct) values 37.2, 37.3, and 34.9) and two on urine collected post travel (Ct values 28.9 and 37.3). The remaining four cases were diagnosed by seroconversion of ZIKV-specific IgG antibodies, confirmed by VNT (Table 2). ZIKV-infected participants had returned from South America (Colombia, n = 1), Central America (Honduras, Nicaragua and Mexico, n = 6), and the Caribbean (Haiti, n = 2). The median time from arrival in an endemic area to symptomatic ZIKV infection was 13 days (range 4–20 days).

The time at risk was calculated to be 53.0 person-months. The ZIKV incidence rate was 17.0% (95% CI 7.8–32.2; Poisson distribution) per month of travel.

Self-reported exposure and protective measures to prevent mosquito bites and the use of chloroquine as malaria chemoprophylaxis did not differ between ZIKV cases and non-cases (Table 1). All ZIKV cases and 70% of non-ZIKV cases had been vaccinated against yellow fever virus. Forty-six baseline serum samples were available for DENV-IgG antibody testing. Anti-DENV antibodies were detected in the pre-travel samples of three participants, and none of these acquired ZIKV infection during the study period.

Legend (Table 1 opposite page)

OR, odds ratio; CI, confidence interval; F, female; M, male; IQR, interquartile range; NS, not significant.
[a] *Mann-Whitney-U test.*
[b] *Each row represents cases vs. non-cases per travel destination, using the other destinations as a comparator (Fisher's exact test, two-tailed probability).*
[c] *Insect repellent, N,N-diethyl-meta-toluamide (DEET).*
[d] *Malaria chemoprophylaxis: chloroquine, 300 mg base (500 mg salt) orally, once/week).*
[e] *Presence of dengue IgG in baseline serum is an indicator of previous dengue virus or related flavivirus infection;*
note: baseline serum missing for two Zika virus cases (Table 2, cases 7 and 8).

Table 1. Demographic and travel data, risk factors and symptoms of 49 travellers to the Americas, 24 of whom reported symptoms and 9 had confirmed Zika virus infection (including 1 asymptomatic case)

	Total	Zika cases (n=9), n (%)		Non-Zika cases (n=40), n (%)		OR (95%-CI]	p-value
Age (years), median (range)	32 (19-64)	29 (22-41)		37 (19-64)			0.11 [a]
Gender (ratio F:M)	38:11	7:2		31:9		1.0 (0.2-5.6)	NS
Travel destination							
South America	20	1	(11.1)	19	(47.5)		0.06 [b]
Central America	24	6	(66.7)	18	(45.0)		0.29 [b]
Caribbean	5	2	(22.2)	3	(7.5)		0.22 [b]
Travel type							
Tourist	40	6	(66.7)	34	(85.0)		0.34 [b]
Student	3	0	(0.0)	3	(7.5)		1 [b]
Relief worker	6	3	(33.3)	3	(7.5)		0.07 [b]
Travel duration							
Days, median (IQR)	21 (16-197)	22 (21-197)		22 (16-118)			NS
Person-months	67.8	18.8		49.0			
Exposure/ protection		9		40			
Insect bites	42	8	(88.9)	34	(85.0)	1.4 (0.15-13.4)	NS
Insect repellent [c]	46	8	(88.9)	38	(95.0)	0.42 (0.03-5.2)	NS
Protective clothing	29	4	(44.4)	25	(62.5)	0.48 (0.11-2.1)	NS
Camping in open air	19	3	(33.3)	16	(40.0)	0.75 (0.16-3.4)	NS
Bed net	25	7	(77.8)	18	(45.0)	4.3 (0.79-23.2)	NS
Chloroquine use [d]	13	3	(33.3)	10	(25.0)	1.5 (0.3-7.1)	NS
Yellow fever vaccination	37	9	(100)	28	(70.0)	-	
Previous dengue infection [e]	3	0/7	(0.0)	3/39	(7.7)	-	
Symptoms	24	8	(100)	16	(100)		
Time to onset (days), median (range)		13 (4-20)		12 (4-86)			NS
Rash	7	6	(75.0)	1	(6.3)	45 (3.4-594)	
Conjunctivitis	0	0	(0.0)	0	(0.0)	-	
Fever	9	2	(25.0)	7	(43.8)		NS
Arthralgia	3	2	(25.0)	1	(6.3)		NS
Myalgia	6	2	(25.0)	4	(25.0)		NS
Headache	6	4	(50.0)	2	(12.5)		NS
Fatigue	6	3	(37.5)	3	(18.8)		NS
Cough	2	0	(0.0)	2	(12.5)		NS
Throat ache	2	0	(0.0)	2	(12.5)		NS
Abdominal pain	6	2	(25.0)	4	(25.0)		NS
Diarrhoea	17	5	(62.5)	12	(75.0)		NS
Constipation	1	0	(0.0)	1	(2.5)		NS
Nausea	8	2	(25.0)	6	(37.5)		NS
Vomiting	7	1	(12.5)	6	(37.5)		NS

Table 2. Demographic and travel data, characteristics, and diagnosis of nine Zika cases in the case-cohort study of travellers to the Americas, 2016

Case	Age (years)	Gender	Destination	Travel Type [a]	Travel Duration (days)	Symptoms	Time to symptom onset (days)	ZIKV RT-PCR [b]	Ct-value	ZIKV ELISA IgG [c]	ZIKV ELISA IgM [c]	ZIKV NT [d]
1	30	F	Colombia	T	22	Yes	12	BFP	37.2	POS	POS	243
2	41	F	Honduras	R	76	Yes	11	BFP	34.9	POS	POS	640
3	31	M	Haiti	R	169	Yes	20	BFP	37.3	POS	NEG	640
4	29	M	Nicaragua	T	21	Yes	19	urine	37.3	POS	NEG	416
5	28	F	Nicaragua	T	21	Yes	19	urine	28.9	POS	NEG	152
6	30	F	Mexico	T	22	Yes	14	.		POS	NEG	640
7	22	F	Mexico	T	22	Yes	9	.		POS	NEG	416
8	24	F	Mexico	T	22	Yes	4	.		POS	NEG	416
9	29	F	Haiti	R	197	No	NA	.		POS	NEG	640

F, female; M, male; ZIKV, Zika virus; Ct, cycle threshold.

a *Travel type: T denotes tourism, R denotes relief work.*

b *Real-time reverse transcription PCR (RealStar Zika Virus RT-PCR Kit, Altona diagnostics GmbH, Hamburg, Germany). The matrix for RT-PCR is reported in the column (BFP = capillary blood on filter paper, collected during illness abroad).*

c *ZIKV IgG/IgM enzyme linked immunosorbent assay (ELISA) (Euroimmun, Lübeck, Germany).*

d *In-house Zika virus neutralization testing assay, 90% neutralizing antibody titre.*

Discussion

This prospective cohort study indicated that adult participants who travelled to areas with epidemic vector-borne transmission were at high risk of acquiring ZIKV infection. The calculated incidence rate of 17.0% per month of travel during the 2016 outbreak in the Americas exceeded surveillance-based estimates of all travel-associated illness frequencies, except for travellers' diarrhoea (12). ZIKV cases in this study cohort had visited Colombia, Honduras, Mexico, and Nicaragua, at a time when the respective national health authorities reported more than 200 (suspected and confirmed) ZIKV cases per epidemiological week (EW) to the Pan American Health Organization (PAHO) (Figure 1)(5). The detection of ZIKV-RNA on BFP, collected by a symptomatic traveller to Haiti in EW 39 (Fig. 1E), confirmed ongoing transmission of ZIKV beyond EW 33, when Haiti sent its last update to PAHO (5,13). This finding highlights the role of travellers as sentinels for ZIKV circulation (14). It also illustrates the utility of BFP, which allowed us to obtain a molecular diagnosis of ZIKV in three cases. In addition, it permitted the time from arrival to symptom onset to be established in symptomatic ZIKV infections, even when the duration of travel was much longer (Table 2 and Fig. 1F, cases 2 and 8).

Only one of nine ZIKV infections was asymptomatic. The sensitivity of the NS1-based ELISA has been debated, but seroconversion for ZIKV-specific IgG is very likely at 20 days post travel-associated exposure (15–17). Given the estimates of symptomatic-to-asymptomatic ZIKV infection ratios in non-traveller populations in Micronesia and French Polynesia (1:4.4 and 1:1, respectively) (18,19), a higher frequency of asymptomatic infection had been anticipated. However, several studies have already suggested that asymptomatic ZIKV infections occur in only 0-27% of non-pregnant returning travellers (20–22).

In line with other series, it was confirmed that skin rash is a predominant feature and this was the only clinical predictor of ZIKV infection in travellers (6,23).

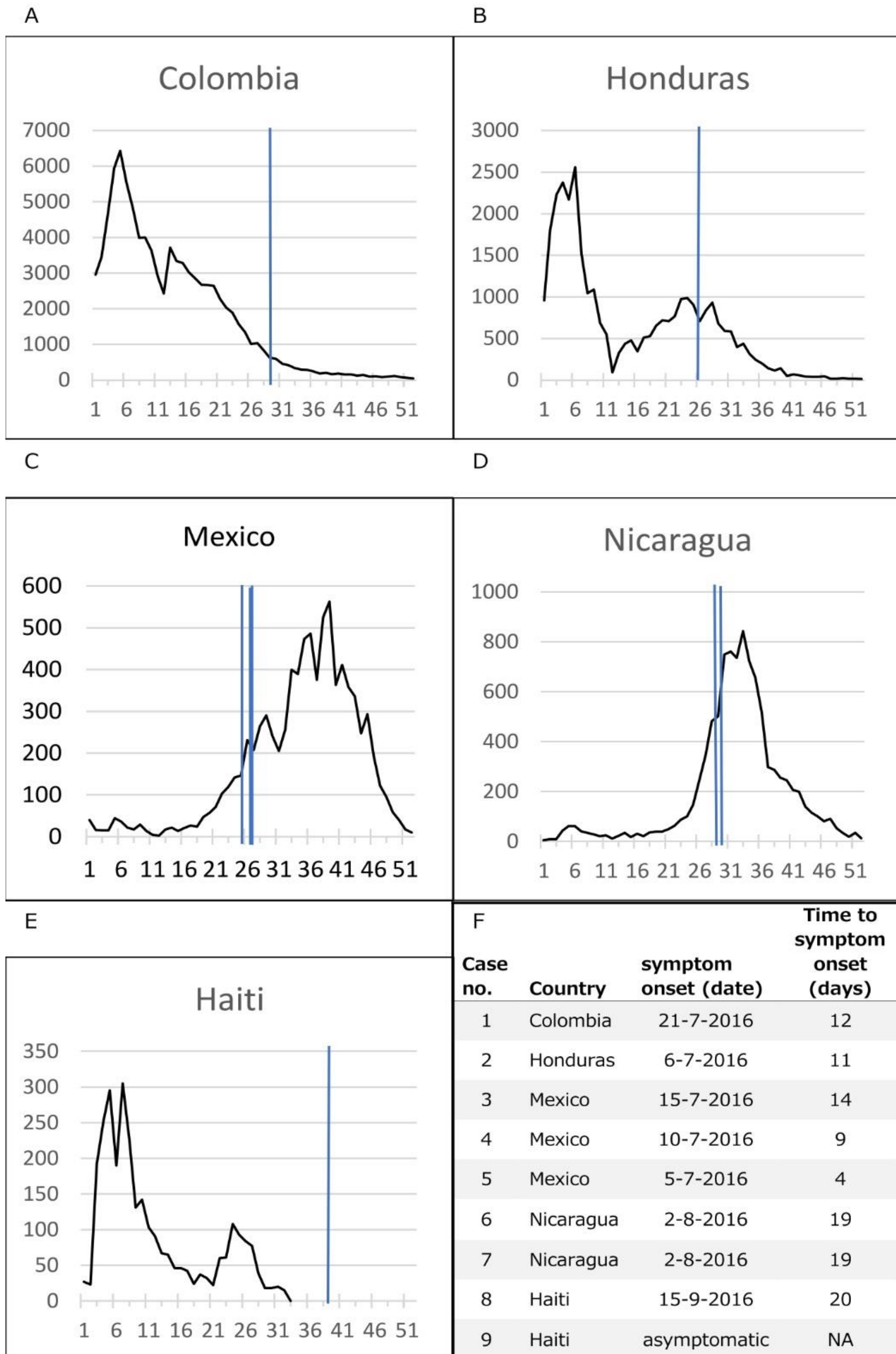
Figure 1. **Countries and timelines of ZIKV infection among participating travellers in relation to the number of autochthonous cases reported to PAHO by the national health authorities (2016)**

Legend Figure 1 A-F (opposite page)

The panels A-E show the epidemic curves of ZIKV infection per country, based on the counts of suspected and confirmed cases (vertical axes) per Epidemiological Week (EW) in 2016 (horizontal axes), as reported to the Pan American Health Organization (PAHO). Note: the scales of the vertical axes are different between panels.

The vertical lines in the graphs indicate the timing of ZIKV infection in symptomatic travellers who participated in our study. Panel F presents the country, date of symptom onset, time from arrival in the country to symptom onset and mode of diagnosis for ZIKV cases in our cohort.

Source: PAHO - Countries and territories with autochthonous transmission in the Americas reported in 2015-2017 (http://ais.paho.org/phip/viz/ed_zika_countrymap.asp);
Note: The latest update on the Zika epidemic PAHO received from the Haiti national authorities was from EW 33 of 2016. However, vector-borne transmission of ZIKV was ongoing beyond EW 33, as reported by Journel et al. (ref. 13).



Case no.	Country	symptom onset (date)	Time to symptom onset (days)
1	Colombia	21-7-2016	12
2	Honduras	6-7-2016	11
3	Mexico	15-7-2016	14
4	Mexico	10-7-2016	9
5	Mexico	5-7-2016	4
6	Nicaragua	2-8-2016	19
7	Nicaragua	2-8-2016	19
8	Haiti	15-9-2016	20
9	Haiti	asymptomatic	NA

Persistent adherence to mosquito bite prevention measures could not be ascertained, but the data suggest that these did not protect against ZIKV infection. However, the study lacked power to draw conclusions in this regard. Chloroquine has shown antiviral activity to ZIKV in various cell models (24). When taken as malaria chemoprophylaxis, chloroquine (dosage of 300 mg base orally, once a week) did not seem to protect against ZIKV infection in three participants.

The strengths of this study are the prospective design, the collection of data and BFP during travel, the availability of follow-up sera for asymptomatic patients, and the use of RT-PCR and VNT-assays to confirm the ZIKV diagnosis. The study also has several limitations. Most importantly, the sample size was small because of the sharp decline in cases towards the end of 2016. Secondly, the risk of acquiring a vector-borne disease is governed by many factors. The analysis was restricted to travellers to countries that reported epidemiological updates on the ZIKV outbreak to PAHO. It is important to be aware that this is an oversimplification of the dynamic and variable risk factors (geographic, climate, population density, activities, etc.) for acquiring vector-borne disease during travel.

In conclusion, the timing of recruitment into an observational cohort study presented the unique opportunity to study the incidence and characteristics of ZIKV infection in adult travellers prospectively. The incidence rate of ZIKV in the study participants who travelled to the epidemic territories during the 2016 outbreak was estimated at 17% per month of travel. It exceeded most other known travel hazards. In retrospect, this finding justifies the precautionary measures and travel restrictions recommended for persons at risk of complications of ZIKV infection, including pregnant women (25,26). Most cases of ZIKV infection were symptomatic, with skin rash as the only predicting symptom. Asymptomatic ZIKV in adult travellers from non-endemic areas may occur less frequently than previously thought.

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Supplementary Material

Incidence of Zika virus infection in a prospective cohort of Belgian travellers to the Americas in 2016

Ralph Huits MD [1], Dorien Van Den Bossche MSc [1], Kaat Eggermont BSc [1], Erica Lotgering BSc [1], Anne-marie Feyens BSc [1], Idzi Potters BSc [1], Jan Jacobs MD PhD [1,2], Marjan Van Esbroeck MD [1], Lieselotte Cnops PhD [1#], Emmanuel Bottieau MD PhD [1#]

[1] Department of Clinical Sciences, Institute of Tropical Medicine, Antwerp, Belgium

[2] Department of Microbiology and Immunology, University of Leuven, Leuven, Belgium.

[#] These authors contributed equally to this manuscript

Key words: Zika virus; outbreak; Americas; incidence rate; prospective cohort study; Enzyme Linked Immunosorbent Assay; virus neutralization testing; real-time reverse transcription polymerase chain reaction; filter paper

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I. Contents of the study envelope, issued to all participants before travel.

Figure 1A.
Content of the study envelope

- study information for participants
- a structured questionnaire
- printed instructions how to collect capillary blood on filter paper (BFP) in the event of fever or skin rash occurring during travel.
- Plastic bag with materials for collection of capillary blood on filter paper.

Figure 1B.
Content of plastic bag:

- 3 silica gel bags
- two alcohol wipes
- 1 humidity indicator
- yellow auto-retractable lancet (Safety-Lancet Extra 18G, Sarstedt AG&Co., Nümbrecht, Germany)
- 1 Whatman 903 filter paper card.

Figure 2. Whatman 903TM filter paper card.

The card contains a filter paper with 5 pre-printed circles, and a cover to make note of the patient identification (study number, initials) and date.

Figure 2A.
Filter paper before use.

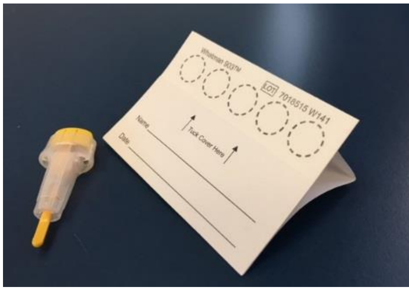
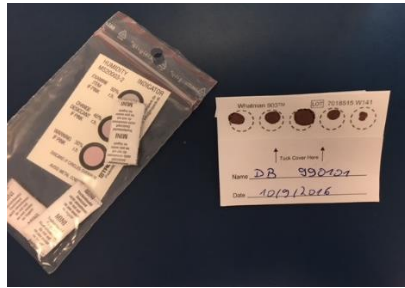


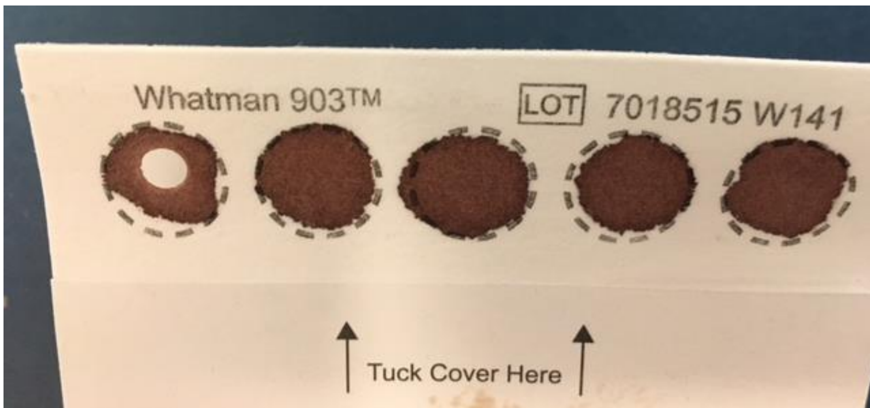
Figure 2B.
Filter paper as received in the laboratory.



II. Laboratory procedures for extraction and detection of ZIKV RNA.

For the RNA extraction, one punch of 5 mm was cut out with a puncher from the filter paper cards that were stored at room temperature in the plastic bag with silica gel and a humidity indicator (Figure 3). One punch contains a volume of ~ 10 µL capillary blood sample. The puncher was decontaminated between different samples by successive immersion for at least 5 sec in the following solutions: 5% sodium hypochlorite, distilled water, distilled water, 70% ethanol and dried by punching a dry filter paper.

Figure 3. One punch cut out from the first spot of capillary blood collected onto the Whatman 903™ filter paper card.



The QIAamp® Viral RNA kit (Qiagen®) was used for optimization of the RNA extraction procedure. After collecting one punch in a 1.5 mL Eppendorf tube, 560 µL AVL buffer and 5,6 µL carrier RNA was added, together with 10 µL internal extraction control (PDV, Phocine Distemper Virus). The sample was vortexed for 15 sec and briefly centrifuged. After an incubation of 10 min at room temperature and a brief centrifugation step, 630 µL of the lysate was transferred into a QIAamp spin column. The filter paper was not transferred to the column. All next steps (washing/elution steps) were performed according to manufacturers' instructions. RNA was eluted from the

column with 80 µL (2 times 40 µL) elution buffer. Automated extraction with the Maxwell RSC (Promega) was used on the clinical BFP samples. One punch of BFP was added to 250 µL COBAS buffer (Roche), 300 µL of lysis buffer (Maxwell kit) and 30 µL proteinase K (Maxwell kit). After vortexing, the sample was incubated for 10 minutes @ 56°C and processed using the Maxwell RSC viral kit (AS1330) according to the manufacturer's instructions.

For ZIKV RNA detection, 10 µL RNA was tested with the commercial available Zika virus-specific RT-PCR kit (RealStar® Zika virus RT-PCR, Altona diagnostics GmbH, Hamburg, Germany) according to the manufacturers' instructions on the LightCycler 96 device (Roche Diagnostics, Basel, Switzerland). The PCR program consists of a RT reaction of 20 minutes at 55 °C and a denaturation step of 2 minutes at 95 °C, followed by 45 cycles of 15 seconds at 95 °C, 55 seconds at 58 °C and 15 seconds at 72 °C. We expressed RNA levels as threshold cycle values (Ct-values), because a reference method for RNA quantification is not yet available. Any Ct-value below 45 is defined as positive. If the sample was negative with the ZIKV RT-PCR, a RT-PCR targeting phocine distemper virus (PDV) was performed to check for PCR inhibition.

III. Preliminary data on optimization of the BFP extraction procedure.

1) The RNA extraction from BFP (n=8, blotted with whole blood spiked with DENV) was performed with the Qiagen viral RNA extraction method and with an elution protocol. The Qiagen extraction was selected as method as it performed better (average Ct-value was 8.86 lower) than with elution procedure.

2) The Qiagen extraction was evaluated on one or two punches from BFP ((n=5, blotted with whole blood spiked with DENV). Differences in observed Ct-values from one or two punches were not significant (≤ 1 Ct-value difference), and for practical reasons and because sometimes the blood spots are small, it was decided to work with one punch.

3) The manual Qiagen extraction was compared to the automated extraction with the Maxwell automate on BFP (n=2, whole blood sample). The automated extraction was more sensitive. The BFPs extracted with the Maxwell automate were positive (Ct-value ~ 37), while the Qiagen extraction was negative for these two BFP.

Preliminary data on the performance of the filter paper (FP) method.

RT-PCR results from the conventionally collected sample and from the FP samples were compared for clinical samples positive for CHIKV (n=4); DENV (n=8), and ZIKV (n=8). Ct-values detected from filter papers blotted with serum (CHIKV), whole blood (DENV or semen (ZIKV), were higher than from the conventional clinical sample (see Table 1 and Figure 4). Two samples with a high Ct-value were negative after blotting on the filter paper. These preliminary results indicate a lower sensitivity for FP than the conventional samples with an average increase in Ct-value of 3.84 (for CHIKV); 7.13 (for DENV) and 3.84 (for ZIKV) compared to respectively serum, whole blood and semen samples.

Figure 4. Comparison of cycle threshold-values of RT-PCR on conventional samples (serum, whole blood and semen samples), and samples blotted on filter paper

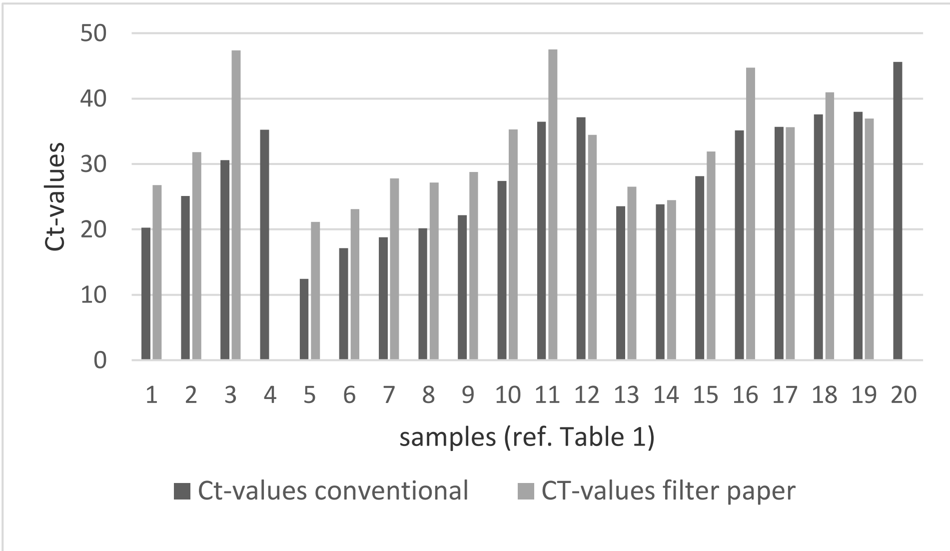


Table 1. Comparison of cycle threshold-values of RT-PCR on conventional samples (serum, whole blood and semen samples), and samples blotted on filter paper.

	target	conventional sample	Ct-value conv. sample	sample blotted on FP	Ct-value FP
1	CHIKV	serum	20,27	serum FP	26,75
2	CHIKV	serum	25,11	serum FP	31,8
3	CHIKV	serum	30,58	serum FP	47,37
4	CHIKV	serum	35,22	serum FP	negative
5	DENV	whole blood	12,45	whole blood FP	21,12
6	DENV	whole blood	17,13	whole blood FP	23,1
7	DENV	whole blood	18,80	whole blood FP	27,78
8	DENV	whole blood	20,19	whole blood FP	27,17
9	DENV	whole blood	22,19	whole blood FP	28,76
10	DENV	whole blood	27,41	whole blood FP	35,3
11	DENV	whole blood	36,44	whole blood FP	47,54
12	DENV	whole blood	37,14	whole blood FP	34,44
13	ZIKV	semen	23,56	semen FP	26,55
14	ZIKV	semen	23,85	semen FP	24,46
15	ZIKV	semen	28,16	semen FP	31,92
16	ZIKV	semen	35,15	semen FP	44,71
17	ZIKV	semen	35,70	semen FP	35,62
18	ZIKV	semen	37,57	semen FP	40,95
19	ZIKV	semen	37,97	semen FP	36,95
20	ZIKV	semen	45,63	semen FP	negative

IV. Calculation of ZIKV infection incidence rate and sensitivity analysis for travellers to the Americas, during the ZIKV outbreak 2016

Incidence rate =
n / (TD – ((TD symp – TTSO symp) + (TD asym– TTI asym)).
n = ZIKV cases, 9 (8 symptomatic, 1 asymptomatic).
(TD – ((TD symp – TTSO symp) + (TD asym– TTI asym)) = time at risk for susceptible travellers.

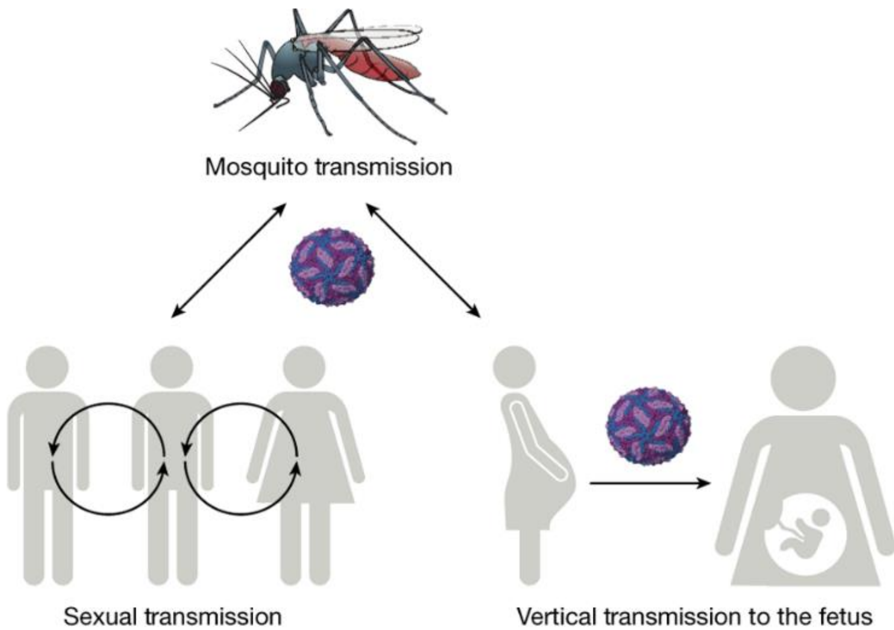
TD (days) *	TD $_{symp}$ (days) †	TTSO $_{symp}$ (days) ‡	TD $_{asym}$ (days)§	TTI $_{asym}$ (days) ¶	Time at risk	Time at risk#	Incidence rate per person-month **	[95%CI] † †
2068	375	108	197	0	1604 (days)	52.6 (months)	17.1 %	[7.8 – 32.5]
2068	**375**	**108**	**197**	**13**	**1617** (days)	**53.0** (months)	**17.0 %**	**[7.8 – 32.2]**
2068	375	108	197	99	1702 (days)	55.8 (months)	16.1 %	[7.4 – 30.6]
2068	375	108	197	197	1801 (days)	59.0 (months)	15.3 %	[7.0-29.0]

**TD = total travel duration for travellers with complete follow-up (n=49).*
†TD symp = total travel duration of symptomatic cases.
‡TTSO symp = total time from arrival in endemic areas to symptom onset (symptomatic cases).
§TD asym = total travel duration of the asymptomatic case.
¶TTI asym = estimated time to ZIKV infection from arrival in endemic areas (asymptomatic case). The TTI asym cannot be ascertained.

We performed a sensitivity analysis for the ZIKV incidence rate if TTI asym equaled 0 days (infection on the day of arrival in endemic areas), 13 days (median time to ZIKV infection for symptomatics), 99 days (infection at the median duration of travel for the asymptomatic case), and 197 days (infection at the day of departure from endemic areas).
#After ZIKV infection, an individual is considered immune to secondary ZIKV infection.
**days are converted to months by dividing the number of days by 30.5.
††Incidence rate estimate (Poisson distribution) with exact 95%-confidence interval.
The incidence rate as calculated with the TTI asym set at 13 days (median time to ZIKV infection for symptomatics), was adopted in the manuscript (bold print in Table).

Chapter 4

The potential for sexual transmission of Zika virus



ZIKV is transmitted in an epidemic cycle between Aedes mosquitoes and humans. ZIKV can also be transmitted through sexual contact or vertical transmission from an infected pregnant mother to her fetus.

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Zika virus in semen: a prospective cohort study of symptomatic travellers returning to Belgium

Ralph Huits, Birgit De Smet, Kevin K. Ariën, Marjan Van Esbroeck, Emmanuel Bottieau, Lieselotte Cnops

Institute of Tropical Medicine, Antwerp, Belgium

Department of Clinical Sciences, Institute of Tropical Medicine, Antwerp, Belgium

Department of Biomedical Sciences, Institute of Tropical Medicine, Antwerp, Belgium

Keywords: zika virus, semen, kinetics, RT-PCR, sexual transmission

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Abstract

Objective

To prospectively monitor Zika viral loads in semen from Belgian travellers with confirmed Zika virus infection, who returned from the Americas during the 2016 Zika virus epidemic.

Methods

We recruited symptomatic travellers consulting our clinic and we confirmed infection with either reverse-transcriptase (RT) polymerase chain reaction (PCR) assay or virus neutralization test. The participants produced semen samples weekly, either at the clinic or at home. For the initial sample, the laboratory staff did a microscopy analysis if they received the sample within an hour of production. Using RT–PCR, we monitored Zika virus ribonucleic acid (RNA) loads in semen until we obtained two negative results.

Findings

We detected Zika virus RNA in nine of 15 participants' semen, one of whom was vasectomized. The median time to loss of RNA detection in semen was 83 days after symptom onset (95% confidence interval, CI: 57–108). The longest duration of viral shedding in semen before obtaining the first negative RT–PCR result was 144 days after symptom onset. All of the 11 participants, for whom we microscopically analysed their semen, had presence of leukocytes, 10 showed haematospermia and six showed oligospermia. These abnormalities occurred irrespective of Zika virus detection in semen.

Conclusion

The majority of men in our study had detectable Zika virus RNA in their semen. We recommend that semen from Zika virus- infected men should be analysed with RT–PCR and that health professionals should advise infected men, even if they are vasectomized, about current recommendations for prevention of sexual transmission of the virus.

Introduction

Zika virus infection in humans may result in a mild disease characterized by rash, fever, arthralgia and conjunctivitis (1,2). However, since its introduction in the Americas in 2015, the Zika virus has been found to cause congenital brain abnormalities and Guillain–Barré syndrome (3). A recent study showed that for pregnant women having symptoms or tested positive for Zika virus in the first trimester, 22 (8%) out of the 276 completed pregnancies had Zika virus-associated birth defects. For second and third trimesters, the numbers were 36 (5%) out of 726 and 20 (4%) out of 494, respectively (4). Therefore, the Zika virus has emerged as a public and reproductive health concern (5). The Zika virus, which belongs to the genus *Flavivirus*, is primarily transmitted by *Aedes* mosquitoes in endemic areas, but the virus can also be transmitted from person-to-person via sexual encounter (6). Evidence exists that people who had not resided in or travelled to areas with arthropod-borne Zika virus transmission have developed the disease after oral, vaginal or anal sexual intercourse with Zika virus infected partners (6–8).

Sexual transmission by asymptomatic men has been reported in a couple seeking assisted reproduction treatment and in a woman with a travelling male sex partner (9,10). The longest documented interval between a man's onset of symptoms and sexual transmission to a woman is 44 days (11). Using reverse-transcriptase (RT) polymerase chain reaction (PCR) assay, researchers have detected Zika virus ribonucleic acid (RNA) in vaginal secretions and semen (12–15). Studies have reported viral shedding in semen beyond 188 days after symptom onset (16). Several studies have assessed Zika virus persistence in semen after acute infection in endemic settings (13,17,18). In a study from Puerto Rico, 56% (31/55) of semen samples from infected men contained viral RNA. The estimated median time until RNA was undetectable in the men's semen was 34 days after symptom onset (95% CI: 28–41) and 95% of semen samples were virus negative after 81 days (95% CI: 64–98) (13). A prospective study from French Guiana found viral RNA in 67% (8/12) of semen samples from infected men (17). The authors concluded that the average persistence of RNA in semen was 26 days, although the

intervals between detection and follow-up sampling were irregular and large (17). A report on 15 infected men from the French oversea territory Guadeloupe, showed that 11 (73%) had viral RNA in semen (18). One study documented viral RNA in semen for 12 out of 23 (52%) British travellers (19). Because of loss to follow-up, the study could only report time to viral clearance in semen for four patients, which ranged from 70 to 132 days after symptom onset. Persistence of Zika virus in immune-privileged sites, such as the male reproductive system, may differ among populations depending on ethnic backgrounds, ongoing vector-borne transmission of the virus and previous exposure to other flaviviruses. Therefore, knowledge of the incidence and kinetics of Zika virus in semen is important for assessing the probability of viral sexual transmission. Here we present the results from a cohort study that prospectively monitored viral loads in semen from Belgian travellers with confirmed Zika virus infection returning from the Americas.

Methods

From February 2016 to May 2017, we tested all travellers who consulted the Institute of Tropical Medicine Antwerp, Belgium, with symptoms matching the European Centers for Disease Control clinical case definition for Zika virus infection – that is, maculopapular rash with or without fever, and painful joints or muscles or non-purulent conjunctivitis (20).

To diagnose Zika virus infection, we first used the anti-Zika virus Immunoglobulin (Ig) M and IgG enzyme linked immunosorbent assay (ELISA; Euroimmun AG, Lübeck, Germany) according to the manufacturers' exact instructions. We confirmed positive or equivocal ELISA results the same week with a non-commercial Zika virus neutralization test on refrigerated serum. To detect viral RNA, we performed commercial available Zika virus-specific RT-PCR (RealStar® Zika Virus RT–PCR Kit, Altona diagnostics GmbH, Hamburg, Germany) according to the manufacturers' instructions on the LightCycler 96 (Roche Diagnostics, Basel, Switzerland).

We tested serum from participants who presented within 7 days after symptom onset or urine within 14 days after symptom onset (21). We extracted RNA from 140 µL serum using QIAamp® RNA viral kit (Qiagen, Hilden, Germany). For urine, we mixed 500 µL with 500 µL cobas® PCR media kit (Roche Molecular Systems, Inc., Pleasanton, United States of America) before extracting RNA with the automated MagNa Pure purification system (Roche Molecular Systems, Inc.). For both serum and urine, we used 10 µL eluates for the RT-PCR assay. The PCR program consists of a RT reaction of 20 minute at 55 °C and a denaturation step of 2 minute at 95 °C, followed by 45 cycles of 15 second at 95 °C, 55 second at 58 °C and 15 second at 72 °C. We expressed RNA levels as threshold cycle values (Ct-values), because a reference method for RNA quantification is not available. Any Ct-value below 45 is defined as positive. We defined confirmed infection as a positive virus neutralization test result or a positive RT-PCR in any clinical sample (22).

Men who were 18 years or older, with a laboratory-confirmed symptomatic Zika virus infection and no history of immunosuppression were eligible to participate in the study. Staff members of the Institute of Tropical Medicine recruited men and recorded clinical and epidemiological data in a standardized case record form (Table 1). Two authors subsequently entered the data in a Microsoft Excel 2010 database (Microsoft Corp., Redmond, USA). For periodic monitoring of Zika virus RNA loads in semen, the participants were instructed to produce semen samples into a sterile cup by masturbation every week. Upon recruitment we provided the participants with materials needed for semen collection. The participants produced the sample at the institute or at home and sent the samples by regular mail at room temperature to the institute. We asked participants to produce semen samples until we obtained two negative results.

If the laboratory staff received the first sample within an hour after production, they analysed the sperm, leukocyte and erythrocyte counts. Oligospermia was defined as a count of less than 15 million spermatozoa/mL (23). For the Zika virus RT–PCR analysis, laboratory staff transferred 140 µL semen into sterile Eppendorf tubes after

liquefaction (30 to 60 minutes after ejaculation) and used QIAamp® RNA viral kit for RNA extraction. The RT–PCR was done as described above. Laboratory staff attempted to isolate the Zika virus from all initial semen samples by inoculating the supernatant of liquefied and centrifuged semen with confluent C6/36 cells growing in Eagle's minimal essential medium with 2% fetal bovine serum. When considerable virus induced cytopathic effects were visible or after one week, the supernatant was passaged on to Vero cells and grown for one week. Laboratory staff monitored virus-induced cytopathic effect and confirmed such effects by RT–PCR.

Statistical analysis

Because of the exploratory character of the study, we arbitrarily set the sample size at 20 participants. For each participant, we calculated the time from symptom onset until Zika virus RNA could no longer be detected in semen. We assessed the duration of Zika virus persistence in semen using the Kaplan–Meier estimator and the parametric Weibull regression models. All analyses were done in R, version 3.3.2 (R Foundation for Statistical Computing, Vienna, Austria).

Ethical approval

We obtained ethical approval from the institutional review board at Institute of Tropical Medicine and the ethics committee of the Antwerp University Hospital, Belgium. We registered the protocol at ClinicalTrials.gov (NCT 02733796).

Results

From February 2016 to May 2017, the institute recruited and followed-up 15 Caucasian men, who had travelled to countries with active vector-borne transmission of Zika virus (Table 1). We did not reach the projected sample size, because of the decline in number of patient with Zika virus infection.

The participants' median age was 45 years (range: 19–65). All men presented with rash; 12 (80%) had fever; eight (53%) had myalgia; six

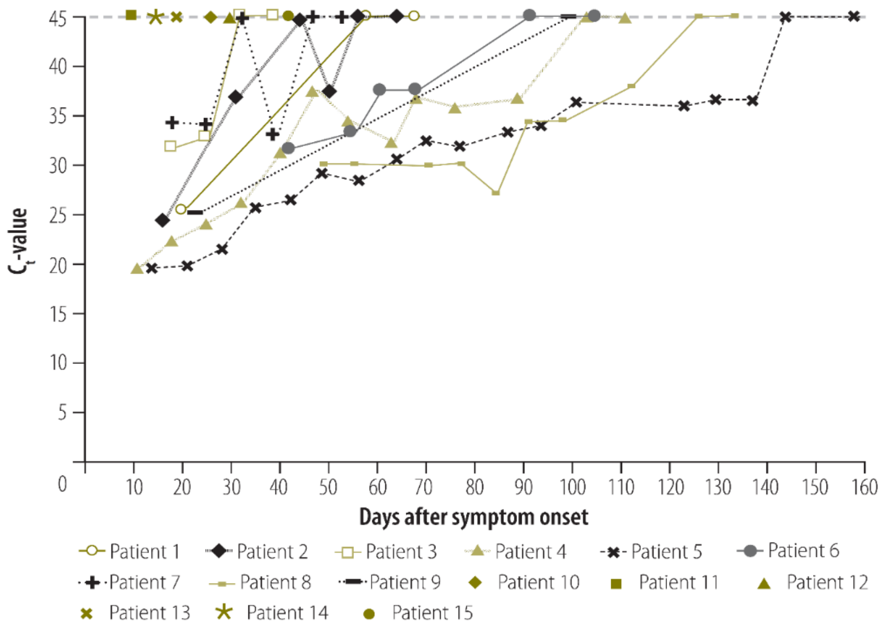
(40%) had headache or retro-orbital pain; five (33%) had arthralgia; and three (20%) had conjunctivitis. Six (40%) men reported diarrhoea and two (13%) described fatigue. The median duration of illness was 5 days (range: 1–10).

RT–PCR analyses confirmed Zika virus infection in 10 men, while virus neutralization tests confirmed infection in five men. All men consented to participate and 13 men completed the study; two patients did not provide enough numbers of semen samples for obtaining two consecutive negative results. Patient 9 provided only two semen samples (at 23 and 100 days after symptom onset) and patient 12 provided only one sample at 30 days after symptom onset, after which he withdrew (no reason given). We detected Zika virus-RNA in the semen from nine men (60%), of which one man has had a successful vasectomy. For patients with RNA in semen, the median number of samples analysed was 6 (range: 2–18). For men with positive RT–PCR result, the median time to collection of the first semen sample was shorter than for men with a negative result, though not significantly (18 days after symptom onset; range: 10–49 versus 23 days after symptom onset; range: 10–42). The longest duration before obtaining the first negative semen RT–PCR result was 144 days after symptom onset, with the last sample testing positive at 137 days after symptom onset (Fig. 1). For patient 7 we observed viral RNA recurrence in semen after a single negative RT–PCR result (Fig. 1). The Weibull distribution curve showed that the median loss of RNA detection in semen occurred at 83 days after symptom onset (95% CI: 57–108). At 149 days after symptom onset (95% CI: 104–194) RNA could no longer be detected in the semen of 95% of the men (Fig. 2).

We managed to isolate the Zika virus from one semen sample, collected on 11 days after symptom onset and with a RT–PCR Ct-value of 19.6. Cytopathic effect appeared only in Vero cells after passage on the C6/36 culture. For 11 patients, the first collected semen sample was available for microscopic analysis. We detected leukocytes in all samples and erythrocytes in nine samples; these included five Zika virus positive and four negative samples (Table 1). For one participant, we found macroscopic haematospermia and detected viral RNA in his semen.

After excluding the vasectomized man (patient 6), six out of 10 participants for whom microscopic analysis of semen was available, had oligospermia. Three of these men had Zika virus positive samples and three had negative samples. Out of the six men with oligospermia, five had reported fever. Only one individual completed the follow-up to monitor normalization of sperm counts.

Fig. 1. Detection of Zika virus RNA in semen from Belgian travellers, by days after symptom onset



RNA: ribonucleic acid; Ct: threshold cycle.

Notes: *We used RealStar® Zika Virus reverse-transcriptase polymerase chain reaction kit (Altona diagnostics GmbH, Hamburg, Germany) for RNA detection. The dashed line represents the cut-off and a Ct-value below 45 is considered positive (detection of RNA) and a Ct-value of 45 is defined as negative. For patients 10, 12, and 15, we did not detect RNA in any sample. Two patients did not complete the study, i.e. obtaining two negative results: patient 9 provided only two semen samples and patient 12 provided only one sample.*

Table 1. Characteristics of men with confirmed Zika virus infection included in the prospective study on Zika virus kinetics in semen, 2016–2017

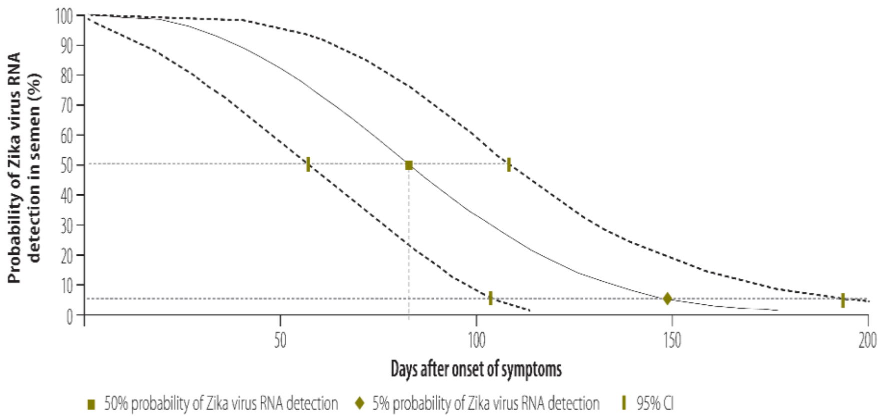
Legend, (table on opposite page)

°	*'R'= rash; 'F'= fever; 'M'= myalgia; 'H'= headache; 'HS'= haematospermia; 'A'= arthralgia; 'D'= diarrhea; 'Fa'= fatigue; 'C'= conjunctivitis* *N/A: not applicable; ND: not determined; RNA: ribonucleic acid; RT–PCR: reverse-transcriptase polymerase chain reaction; VNT: virus neutralization test.*
a	*We managed to isolate the Zika virus from this sample by inoculation of C6/36 cells and Vero cells.*
b	*Patient was vasectomized.*
c	*Recurrence of Zika virus-RNA detection in semen after one negative RT–PCR result at day 32.*
d	*Oligospermia defined as a sperm count < 15 million sperms per mL (ref. 23)*

Notes: *We used RealStar® Zika Virus RT–PCR kit (Altona diagnostics GmbH, Hamburg, Germany) for RNA detection on patient with active infection.*
VNT, a non-commercial test, was used for patients who presented at the clinic after the active viraemic phase.

Patient no.	Age (years)	Travel destination	Date of symptom onset, 2016	Reported symptoms [e]	Duration of illness (days)	Method of initial Zika virus diagnosis (type of sample)	Semen samples						
							Day of First sample, after symptom onset	No. of samples collected	Zika virus RNA detected	Days from symptom onset to first negative RT–PCR	Leukocyte count, cells/µL	Erythrocyte count, cells/µL	Sperm count, million/mL
1	45	Venezuela	6 February	R, F, M, H	6	RT–PCR (urine)	20	6	Yes	58	960	922	8.8[d]
2	33	Haiti	6 February	R, F, M, HS	7	RT–PCR (urine)	16	6	Yes	56	ND	ND	ND
3	38	Guadeloupe	15 July	R, M, H	3	RT–PCR (urine)	18	4	Yes	32	92	4	3.4[d]
4	54	Belize and Guatemala	1 August	R, F, A, M, Fa, D	7	RT–PCR (urine)	11[a]	13	Yes	103	180	280	36.8
5	28	Nicaragua	7 August	R, A, M,H, D	5	RT–PCR (urine)	14	18	Yes	144	102	0	25.2
6	55	Dominican Republic	15 July	R, F	1	VNT (serum)	42	6	Yes	92	1850	72	N/A[b]
7	62	Cuba	30 November	R, F, D	4	RT–PCR (urine)	18	6	Yes	47[c]	40	20	0.4[d]
8	28	Guatemala, Nicaragua	12 December	R, F, H, C	6	RT–PCR (urine)	49	10	Yes	126	ND	ND	ND
9	44	Aruba and Curaçao	13 December	R, F, A, M, C, Fa	6	VNT (serum)	23	2	Yes	100	ND	ND	ND
10	46	Martinique	8 April	R, F	4	VNT (serum)	26	2	No	N/A	252	4	24.1
11	30	Guadeloupe	2 June	R, F, H, D	5	RT–PCR (serum and urine)	10	2	No	N/A	112	12	0.9[d]
12	46	Dominican Republic	4 June	R, F	1	VNT (serum)	30	1	No	N/A	200	44	2.9[d]
13	48	Jamaica	17 July	R, F, A, M, C, D	5	RT–PCR (urine)	19	3	No	N/A	20	0	22.6
14	19	Guadeloupe	22 July	R, F, A, M, H	10	RT–PCR (urine)	15	3	No	N/A	182	14	10.2[d]
15	65	Mexico	25 October	R, D	3	VNT (serum)	42	2	No	N/A	ND	ND	ND

Fig. 2. Estimated time to loss of Zika virus RNA detection in semen



CI: confidence interval; RNA: ribonucleic acid.

Notes: *The Weibull distribution of probability of Zika virus RNA detection in semen assumes 100% probability at the start. We only included participants with detectable Zika virus RNA in semen (n = 9). The solid line represents the median probability of survival; the dashed lines represent the upper and lower margins of the 95% confidence interval.*

Discussion

Here we report on the frequency and persistence of Zika virus in semen after acute symptomatic infection in Belgian men who had travelled to the Americas during the 2016 Zika virus outbreak. In 60% of the men with confirmed Zika virus infection, we also detected Zika virus RNA in the semen. Several studies have reported a similar proportion of detection, both in endemic settings (13,17,18) and in travellers (19). For the six participants with no detectable RNA in semen, we cannot exclude early infection of the male reproductive organs, since others have reported short-lasting presence of Zika virus RNA in semen, as short as 1 day after symptom onset (17). The median time to the first sampling of semen from these six men was 23 days after symptom onset, which suggest that the Zika virus may have already been eliminated from the semen. Therefore, we as well as other researchers might have underestimated the proportion of men with virus in their semen.

Persistence of Zika virus RNA in semen after acute infection appears common. In our study, sequential semen samples showed decreasing level of RNA until the virus became undetectable, confirming previous findings (13,17,19). However, our data showed almost two months longer clearance time than the cohort studies from endemic settings (13,17,18). We cannot ensure that a difference existed in seminal viral persistence between travellers and endemic populations. Both our and other studies may have underestimated the duration of viral shedding in patients with longer semen sampling intervals or in patients having only one negative RT–PCR result (13,17,19). We could exclude differences in diagnostic sensitivity since we used the same RT–PCR assays as two of the other studies (17,18). It is also unlikely that the differences can be attributed to ethnic background of the host, as the proportion of infected semen samples did not differ across our cohort and the endemic studies. One possible explanation could be the difference in natural acquired immunity or vaccination status between men residing in endemic areas and travellers. People living in endemic areas are exposed to other circulating flaviviruses such as dengue, which may affect their ability to clear the virus. In our cohort, we could not assess previous exposure to dengue virus, since the antibody detection assays cross-react with the Zika virus antibodies.

The decline of Zika virus-RNA levels in semen observed in our patients suggests elimination of the virus. In only one patient, we could detect viral RNA after one negative RT–PCR result, but who subsequently had two consecutive negative samples that concluded followup. This finding demonstrates the need for two consecutive negative results, since the possibility of viral recurrence cannot be excluded. Almost all men who had their semen analysed showed macroscopic or microscopic haematospermia. Other studies have also investigated haematospermia following Zika virus infection, but the symptom has not been consistently found (11,14,16,24). The observation of inflammatory cells in semen, irrespective of Zika virus RNA detection, may indicate some degree of tissue damage to the male reproductive tract in the majority of infected men. The presence of erythrocytes, leucocytes and oligospermia in semen could be a sign of inflammation and disruption of the tight-junctions between Sertoli cells that form the blood-testis barrier (25). In a mouse model, Zika virus infection resulted in

histological injury to Sertoli and Leydig cells and to the lumen of the epididymis, and the infection was associated with reduced levels of inhibin β and testosterone (25). Recently, a human study demonstrated that men with active Zika virus infection had increased follicle stimulating hormone concentrations, while they had decreased inhibin β and testosterone concentrations, and median sperm counts (18). The presumed target cells for Zika virus replication in the male reproductive tract are therefore likely to include the seminiferous epithelium. Interestingly, the duration of Zika virus shedding in semen in our cohort may coincide with the time required for spermatogonial renewal, differentiation and proliferation in humans (reported to be 74 days; 95% CI: 69–80) (26). Some of the participants had oligospermia, which could result from inflammatory destruction of the seminiferous epithelium directly, from the fluctuation of reproductive hormone concentrations or from the febrile state (18).

As reported previously, presence of viral RNA in the ejaculate of the vasectomized participant suggests that the Zika virus does not only infect spermatogonia, but also tissues distal of the vasectomy site (27,28). Isolation of the virus is regarded as a proxy for infectivity. However, we were only able to isolate the Zika virus from one of our patients' semen. The failure to isolate the virus from the other patients could be an indication of viral degradation, although others have demonstrated Zika virus replication competence in semen up to 69 days after symptom onset (28). Lower viral loads or laboratory conditions may explain why virus isolation was not more successful (29,30). However, as long as the virus can be detected in semen by RT-PCR, we do not exclude that the virus can be sexually transmitted.

Zika virus in semen may become the major transmission route in areas where arthropod vectors do not thrive. However, mathematical modelling suggests that sexual transmission alone is not likely to drive or sustain a Zika virus outbreak in absence of a suitable vector population (31). Concerns remain that sexual transmission of Zika virus to pregnant women may increase the risk of poor neonatal outcomes in addition to vector-borne infection alone. In mouse models, vaginal infection during pregnancy led to restriction of fetal growth and to fetal brain infection (32,33).

Our study has at least three limitations. First, we had to discontinue the recruitment of participants before reaching the projected sample size, because incidence of Zika virus infection had declined by May 2017 (34). The relatively small sample size limits the generalizability of our results. Second, the follow-up period after obtaining two negative RT–PCR results in semen samples was relatively short. This time may have been too short to detect recurrence of RNA in semen. Third, we could not ascertain the recovery of sperm counts in men with oligospermia. Production of fresh samples suitable for microscopic semen analysis at the study site, proved too demanding for the participants.

The findings presented here emphasize that further studies are needed to increase our understanding of the host-pathogen relationship and implications of Zika virus infection for reproductive function. Pending new evidence, we recommend the use of RNA detection assays for semen of returning travellers with confirmed Zika virus infection, especially for couples planning a pregnancy. To reduce the risk of sexual transmission of the virus, our findings highlight that health professionals should advice patients, even vasectomized men, about the current recommendations from the World Health Organization and United States Centers for Disease Control and Prevention (35,36).

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We submitted the preliminary results of patient 1, 2, 10, and 11 to the Zika Open platform at the Bulletin of the World Health Organization website and the manuscript was published online 6 July 2016 (15).

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Chapter 5

Zika virus infection in returning travellers

You've thrown the worst fear

That can ever be hurled

Fear to bring children

Into the world

Bob Dylan (1963)

A cross-sectional analysis of Zika virus infection in symptomatic and asymptomatic non-pregnant travellers: experience of a European reference center during the outbreak in the Americas

Ralph Huits [1], Ula Maniewski [1], Dorien Van Den Bossche [1], Erica Lotgering [1], Achilleas Tsoumanis [1], Lieselotte Cnops [1], Jan Jacobs [1,2], Marjan Van Esbroeck [1], Emmanuel Bottieau [1]

[1] Institute of Tropical Medicine Antwerp (ITM), Belgium

[2] Department of Microbiology and Immunology, KU Leuven, Belgium

Key words: Zika virus, outbreak, returning traveller, asymptomatic, ELISA, likelihood ratio

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Abbreviations:

95%-CI	95% confidence interval
95%-CrI	95% credibility interval
CAM	Central America
CAR	Caribbean
DENV	dengue virus
DPSO	Days post symptom onset
ELISA	Enzyme Linked Immunosorbent Assay
IQR	Inter-quartile range
ITM	Institute of Tropical Medicine, Antwerp, Belgium
JEV	Japanese encephalitis virus
LRN	Negative likelihood ratio
LRP	Positive likelihood ratio
RT-PCR	Real-time reverse transcription polymerase chain reaction
SAM	South America
TBEV	tick-borne encephalitis virus
VFR	Visiting Friends and Relatives (travel type)
VNT	virus neutralization testing
WNV	West Nile virus
YFV	yellow fever virus
ZIKV	Zika virus

Abstract

Background

Zika virus (ZIKV) infection is a concern to travellers because of potential sexual transmission and adverse pregnancy outcomes.

Objective

To describe our experience in diagnosing ZIKV in travellers returning from endemic territories.

Method

Travellers were evaluated for ZIKV at our clinic in a 12-month period during the outbreak, using ZIKV-specific RT-PCR and anti-ZIKV immunoglobulin M/G ELISA when symptomatic, and ELISA only for asymptomatic travellers, preferably from 20 days after the last exposure. All positive ELISA results were subject to confirmation by Virus Neutralization Testing. We estimated post-test probabilities of ZIKV in asymptomatic travellers.

Results

Of 462 travellers, 227 reported symptoms and 235 did not. Asymptomatic travellers had similar baseline characteristics, but were younger (median age 33 vs. 37 years, p=0.01) and had reproductive concerns more often (75.8% vs. 24.2%). ZIKV infection was confirmed in 49 cases: 46/227 (20.3%) were symptomatic and 3/235 (1.3%) asymptomatic. Rash (positive likelihood ratio (LRP) 5.6) and conjunctivitis (LRP 10.8) predicted ZIKV infection. The post-test probability of a negative ELISA-result at 20-25 days was below 0.1%.

Conclusion

ZIKV infection was frequent in symptomatic, but not in asymptomatic travellers. We consider negative ELISA results at 20-25 days after exposure a safe strategy to rule out ZIKV infection. Testing for ZIKV-specific antibodies within this timeframe could be particularly valuable in the management of returning travellers who wish to conceive.

Introduction

Travellers to areas with vector-borne Zika virus (ZIKV) transmission are at risk of infection (1). Clinically manifest ZIKV infection usually presents as a mild dengue-like illness. However, exposed travellers perceive ZIKV infection as a serious hazard because of secondary sexual transmission or its association with ZIKV congenital syndrome (2,3). These considerations sparked an increasing demand for ZIKV diagnostics even in the absence of symptoms, as approximately 80% of cases in endemic settings were reported to be asymptomatic (4–6). Many sought medical advice and needed counseling about the risk of sexual transmission and adverse pregnancy outcomes after travel-associated exposure to ZIKV.

The evidence to support health care providers in meeting this demand has been scarce. To enable travellers to make informed choices, two important challenges need to be addressed. First, knowledge of the natural history of ZIKV infection and its implications for sexual transmission is incomplete. Regularly updated guidance documents issued by the World Health Organization and the Centers for Disease Control have been available since February 2016, which advocate safer sex practices or abstinence for two months after exposure for women and six months for men to reduce the risk of sexual transmission of ZIKV for people living in regions without active ZIKV transmission (7,8). These timeframes were based on estimates of the maximum duration from symptom onset in the index case to sexual transmission of ZIKV, reportedly up to 41 days post symptom onset (DPSO) (9).

The second challenge is presented by a combination of unknown prevalence of ZIKV in the travellers population, uncertainty over the diagnostic performance of new molecular and serological assays, and incomplete understanding of ZIKV kinetics and the ZIKV-specific antibody response (10). Individual case management should be based on the use of appropriate tools that assist physicians in translating pre-test into post-test probabilities. We made assumptions to develop a diagnostic approach for returning travellers at the Institute of Tropical

Medicine, Antwerp (Belgium) (ITM). At early stages of the ZIKV outbreak in the Americas, we estimated the performance characteristics of the Enzyme Linked Immunosorbent Assay (ELISA) employed at ITM, from available published data. A model of seroconversion rates per DPSO was adopted to estimate the sensitivity of anti-ZIKV antibody detection (11). To estimate post-test probabilities for travellers with unknown prevalence of ZIKV infection, particularly when asymptomatic and seeking to rule out a diagnosis, likelihood ratios can be used (12,13). Here, we report on our experience with this practical diagnostic approach.

Methods

We conducted a cross-sectional observational study of all travellers screened for ZIKV at the travel clinic of the ITM from February to November 2016. Inclusion criteria were age (18 years or older) and possible exposure to ZIKV infection. Exposure was defined by travel to or residence in areas with reported vector-borne transmission of ZIKV (14), restricted to South America (SAM) and Central America (CAM) including the Caribbean (CAR), and truncated at 18 months preceding consultation (i.e. exposures since May 1, 2015). Chart review was performed to retrieve the following data: region of exposure, duration of travel, type of travel (holiday, business, visiting friends and relatives (VFR), or residents (defined as travelling for more than 6 months), reproductive concerns (wish to conceive < 1 year, future wish to conceive, pregnancy or assisted reproductive treatment), presence and nature of symptoms. We also recorded the timing from symptom onset or last exposure to laboratory analysis, and outcome of ZIKV diagnostics. The European Centers for Disease Control (ECDC) Clinical Case Definition (Case Definition) defined ZIKV infection by the presence of at least two of the following symptoms: a maculopapular rash with or without fever, and painful joints or muscles or non-purulent conjunctivitis (15).

Asymptomatic adults wishing to conceive were recommended to test for ZIKV antibodies from 20-30 days after the last exposure. Detection of Immunoglobulin (Ig) M and G was performed using an anti-ZIKV

IgM/IgG Enzyme Linked Immunosorbent Assay (ELISA) (Euroimmun, Lübeck, Germany) according to the manufacturers' instructions. All positive or equivocal anti-ZIKV IgM/IgG results were considered diagnostic only when confirmed by ZIKV Virus Neutralization Testing (VNT). For VNT, patient serum dilutions were incubated with ZIKV strain MR766 at 37°C, and then used to inoculate a Vero cell-line (ATCC® CCL-81™) in a 96-well format at 37°C/7% CO2 for 7 days. The wells were then assessed for virus-induced cytopathic effect. A reciprocal VNT Ig titer of >1/10 was considered positive. In addition to ELISA, symptomatic travellers were tested with ZIKV-specific real-time reverse transcription polymerase chain reaction (RT-PCR) on serum samples upon presentation within 7 DPSO and on urine within 14 DPSO (16). During the study the more sensitive RealStar® Zika Virus RT-PCR Kit (Altona diagnostics GmbH, Hamburg, Germany) replaced a previously described in-house assay (17). A semen sample for RT-PCR was requested from men with molecular or serologic evidence of ZIKV.

The positive likelihood ratio (LRP) of symptoms and of a match with the Case Definition for ZIKV diagnosis in our cohort of symptomatic travellers was calculated, using the formula LRP=probability ('symptom'/'ZIKV cases')/ probability ('symptom/'non-cases'). To calculate the negative likelihood ratio (LRN=(1-sensitivity)/specificity') in our cohort, we made the following assumptions. Because of paucity of data on the accuracy of ZIKV tests, sensitivity for ELISA (IgM and IgG) was set equal to published estimates of seroconversion to ZIKV infection at 15, 20 and 25 DPSO (source data courtesy of Lessler) (11). These estimates were 98.4% (95%-Credible Interval (95%-CrI) [77.2-100]), 100% (95%-CrI [92.6-100]) and 100% (95%-CrI [97.8-100]), respectively. The reported analytic specificity of the Euroimmun ZIKV IgG ELISA was 100% (95%-confidence interval (CI) [95.9–100.0]), and specificity of the ZIKV IgM ELISA was 97.5% (95%-CI [93.0-99.1]) (18, 19). The LRN was calculated using the lower margins of the 95%-CrI for seroconversion at 15, 20 and 25 DPSO. With 95% certainty, the corresponding LRNs (conservative LRN) were lower than 0.24, 0.08 and 0.02 with specificity set at 95%, and 0.23, 0.07 and 0.02 with specificity at 100% (Fig. 1). The post-test probabilities (P1) for asymptomatic travellers of not having

ZIKV infection after a negative ELISA test result were calculated in our study population (pre-test probability (P0)), using the formulas: Pre-test odds = P0/ (1 – P0); Post-test odds = Pretest odds * LRN; P1= Posttest odds / (Posttest odds + 1) (12)(13).

Statistical analysis.

Counts and percentages were calculated for categorical variables, and medians and inter-quantile ranges for continuous variables. Asymptomatic persons were compared with symptomatic patients using the Wilcoxon rank sum test for continuous characteristics and Chi-square or Fisher's exact test for categorical characteristics. Travel, clinical and diagnostic data were recorded in MS Excel 2010 (Microsoft Corp., Redmond, WA, U.S.A.). All analyses were done in Stata (StataCorp. 2015. Stata Statistical Software: Release 14. College Station, TX: StataCorp LP).

Ethics statement.

It is ITM's policy that de-identified data of patients consulting ITM's physicians can be used for research unless the patients explicitly state their objection. The Institutional Review Board of ITM approved the institutional policy of this presumed consent as long as patients' identity is not disclosed to third parties. All data have been analyzed anonymously.

Results

From February 2016 to February 2017, 462 persons were screened for ZIKV at ITM, 235 were of female and 227 of male gender. The median age was 32 years (range 18-79 years). All had travelled to SAM (47.2%), CAM (24.5%), or CAR (31.8%) since May 2015. The total travel duration in our cohort, which included residents of the ZIKV-endemic areas was 985 person-months (median 20 days, inter-quartile range (IQR) [12-37]). Two hundred thirty-five travellers were asymptomatic, and 227 reported symptoms. The mean age in the asymptomatic group was lower than that in symptomatic travellers (32.8 vs. 37 years, $p= 0.01$). Hundred seventy-five asymptomatic (74.5%) vs. 55 symptomatic travellers (24.2%) expressed reproductive concerns (Table 1).

We diagnosed 49 cases of ZIKV infection, 46 of 227 symptomatic (20.3%, 95%-CI [15.6-25.6]) and 3 of 235 asymptomatic travellers (1.3%, 95%-CI [0.4-3.7]). Cases were older (median age 38 vs. 31 years, (p 0.01)). Zika cases and non-cases had equal gender distribution. The travel type was holiday in 49.0% of cases vs. 75.3% in non-cases, business in 16.3% vs. 15.3%, VFR in 6.1% vs. 3.6% and residency in 28.6% vs 5.8% (Table 2). The 3 asymptomatic cases were native to ZIKV-endemic countries (Haiti and Mexico) and had serologic evidence of prior exposure to *Flavivirus* infections.

Eight cases (3.7%) had travelled to SAM, 23 (20.4%) to CAM and 22 (15.0%) to CAR. The median duration of travel was longer for ZIKV-cases (29 days, IQR [18-264] vs. 19 days IQR [12-32], p=0.06), but the difference was not significant, particularly after exclusion of the 'resident' travel type (Table 2).

The first day of illness was recorded for 188 of symptomatic travellers, including 43 ZIKV cases. The median time from symptom onset to laboratory analysis in this group was 19 days (IQR [7-40]). The median time from the last day of travel-associated exposure to testing in symptomatic persons was 17 days (IQR [5-34]) and in asymptomatic 25 days (IQR [20-44]). Fifty-seven asymptomatic travellers (24.3%) were tested prior to the recommended 20 days after their return. ZIKV was confirmed in 2/27 (11.5%) tested at 0-14 days, and in none of 30 tested at 15-19 days after travelling.

Anti-ZIKV-IgM was not detectable in 12 of 18 (67%, 95%-CI [43.7-83.7]) sera with confirmed ZIKV that were sampled later than 20 days after travel-associated exposure. Anti-ZIKV IgG appeared in 14 of 25 sera (56.0%, 95%-CI [37.1-73.3]) that were tested within 15 days after travelling. ZIKV infection was diagnosed by RT-PCR in 20 cases (on serum n=2, urine n=12, serum and urine n=3, semen n=3) and by VNT in 36 travellers. Seven cases were confirmed by both methods. Three of 38 (7.9%) anti-ZIKV IgG and 1of 24 (4%) IgM positive sera were not confirmed by VNT; all had IgG antibodies against dengue virus (DENV). Results of ZIKV diagnostic assays in function of time after travel are presented in Table 3.

Table 1.
Legend (table on opposite page)

* *Eight had visited both SAM and CAM, 5 SAM and CAR, and 4 CAM and CAR*
** *Time from symptom onset to laboratory diagnosis data available for 188 symptomatic travellers*
° *Confirmation = composite diagnosis, ZIKV-specific RT-PCR positive in any sample AND/OR VNT positive*
\# *Sympt. denotes symptomatic travellers; Asympt. denotes asymptomatic travellers;*
Traveltype VFR: Visiting friends/relatives; ART: assisted reproductive treatment

Table 1. **Baseline characteristics of symptomatic and asymptomatic travellers evaluated for ZIKV at ITM after returning from the Americas, February 2016 - February 2017 (n=462)**

Characteristic	Totals (n=462)		Sympt.# (n= 227)		Asympt.# (n=235)		p-value
Age (yrs.), mean, median [IQR]	34.9	32 [28-39]	37.0	33 [28-46]	32.8	31 [28-36]	0.01
Male to female ratio	0.49	(227/235)	0.45	(103/124)	0.53	(124/111)	0.1
Travel type							
Holiday	335	(72.5%)	165	(72.7%)	170	(72.3%)	1
Business	71	(15.4%)	31	(13.7%)	40	(17.0%)	0.4
VFR	18	(3.9%)	9	(4.0%)	9	(3.8%)	0.9
Resident	38	(8.2%)	22	(9.7%)	16	(6.8%)	0.3
Travel destination *							
South America	218	(47.2%)	96	(42.3%)	122	(51.9%)	0.04
Central America	113	(24.5%)	61	(26.9%)	52	(22.1%)	0.3
Caribbean	147	(31.8%)	81	(35.7%)	66	(28.1%)	0.1
Travel duration (total)°							
Person-months	985		579		405		0.06
Days, median [IQR]	20	[12 - 37]	22	[12-60]	19	[12-31]	0.1
0-14 days	166	35.9%	79	(34.8%)	87	(37.0%)	0.6
15-29 days	147	31.8%	65	(28.6%)	82	(34.9%)	0.2
30-89 days	71	15.4%	34	(15.0%)	36	(15.3%)	1
>= 90 days	78	16.9%	48	(21.1%)	30	(12.8%)	0.02
Travel duration (residents excl.)							
Person-months	530	(n=424)	326	(n=205)	204	(n=219)	0.1
Days, median [IQR]	18	[18 – 30]	19	[12-32]	17	[11-28]	0.14
Reproductive concerns							
Any	230	(49.8%)	55	(24.2%)	175	(75.8%)	0.0
Child wish < 1 year	102	(22.1%)	18	(7.9%)	84	(35.7%)	0.0
Future child wish	56	(12.1%)	15	(6.6%)	41	(17.5%)	0.0
pregnant (partner)	59	(12.8%)	17	(7.5%)	42	(17.9%)	0.0
ART	13	(2.8%)	5	(2.2%)	8	(3.4%)	0.6
Time from symptom onset to laboratory diagnosis**			19	[7-40]	NA		
Time from exposure to laboratory diagnosis			17	[5-34]	25	[20-44]	0.0
Zika diagnosis confirmed °	49	(10.6%)	46	(20.3%)	3	(1.3%)	0.0

Table 2. **Epidemiological, clinical and laboratory features of travellers evaluated for Zika virus infection at ITM from February 2016 - February 2017, by diagnosis (n=462)**

Characteristic	Totals		ZIKV cases (n= 49)		Non-ZIKV cases (n= 413)		p-value
Age (yrs.), mean, median [IQR]	34.9	32 [28-39]	39.7	38 [28 – 54]	34.3	31 [28 – 38]	0.01
Male to female ratio	0.49	(227/235)	0.45	(22/27)	0.50	(205/208)	0.55
Travel type							
Holiday	335	72.5%	24	(49.0%)	311	(75.3%)	0.0
Business	71	15.4%	8	(16.3%)	63	(15.3%)	1.0
VFR	18	3.9%	3	(6.1%)	15	(3.6%)	0.42
Resident	38	8.2%	14	(28.6%)	24	(5.8%)	0.0
Travel destination *							
South America	218	47.2%	8	(16.3%)	210	(50.8%)	0.0
Central America	113	24.5%	23	(46.9%)	90	(21.8%)	0.0
Caribbean	147	31.8%	22	(44.9%)	125	(30.3%)	0.05
Travel duration (total)°							
Person-months	985		579		405		0.06
Days, median [IQR]	20	[12 – 37]	29	[18 – 264]	19	[12 – 32]	0.0
0-14 days	166	35.9%	8	(16.3%)	158	(38.3%)	0.0
15-29 days	147	31.8%	18	(36.7%)	129	(31.2%)	0.52
30-89 days	71	15.4%	9	(18.4%)	62	(13.4%)	0.68
>= 90 days	78	16.9%	14	(28.6%)	64	(15.5%)	0.03
Travel duration (residents excl.)							
Person-months	530	(n=424)	35	(n=35)	495	(n=389)	
Days, median [IQR]	18	[18 – 30]	22	[15 – 32]	17	[11 – 30]	0.09
Reproductive concerns							
Any	230	49.8%	9	(18.4%)	221	(53.5%)	<0.001
Child wish <1 year	102	22.1%	1	(2.0%)	101	(24.5%)	
Future child wish	56	12.1%	5	(10.2%)	51	(12.3%)	
pregnant (partner)	59	12.8%	0	0	59	(14.3%)	
ART	13	2.8%	3	(6.1%)	10	(2.4%)	
Symptomatic	227	49.1%	46	(93.9%)	181	(43.8%)	
Asymptomatic	235	50.9%	3	(6.1%)	232	(56.2%)	<0.001

** Eight had visited both SAM and CAM, 5 SAM and CAR, and 4 CAM and CAR*
° travel duration truncated at May 1, 2015
Traveltype VFR: Visiting friends/relatives; ART: assisted reproductive treatment

Table 3. ZIKV diagnostic assay results in function of time after end of travel/exposure

time after travel		ZIKV infection (n= 49)				No ZIKV infection (n=413)			
	Assay*	positive	negative	ND	tested	positive	negative	ND	tested
0 - 14 days	IgM	15	10	3		0	100	7°	
	IgG	14	11	3		1	99	7°	
	VNT	15	0	13		0	2	105	
	RT-PCR	18	4	6	28	0	31	76	107
15 - 19 days	IgM	3	0	0		0	40	0	
	IgG	3	0	0		0	40	0	
	VNT	3	0	0		0	0	40	
	RT-PCR	1	0	2	3	0	3	37	40
20 - 24 days	IgM	3	2	0		1	79	0	
	IgG	5	0	0		0	80	0	
	VNT	5	0	0		0	2	78	
	RT-PCR	1	0	4	5	0	0	80	80
> 25 days	IgM	3	10	0		0	186	0	
	IgG	13	0	0		2	184	0	
	VNT	13	0	0		0	3	183	
	RT-PCR	0	2	11	13	0	2	184	186

** IgM and IgG: anti-ZIKV IgM/IgG Enzyme Linked Immunosorbent Assay (ELISA) (Euroimmun, Lübeck, Germany); RT-PCR: RealStar® Zika Virus RT-PCR Kit (Altona diagnostics GmbH, Hamburg, Germany) and/or an in-house assay (De Smet et al.) (17); VNT: virus neutralization testing.*

ND denotes not done.

° ELISA was not performed in 7 RT-PCR negative samples, up to 7 days post travel

The three asymptomatic travellers had anti-ZIKV antibodies (IgG, but not IgM), and a positive VNT. The first, a 44-year old female VFR traveller who had visited her family in Venezuela in January 2016 and tested 56 days after exposure. She was the partner of a RT-PCR confirmed symptomatic case. She had anti-DENV IgG antibodies and a positive Plaque Reduction Neutralization Test indicating previous infection with DENV-2. The second, a 37-year old male resident of Haiti with child wish, was tested 9 days after travelling to Belgium. Anti-DENV IgG antibodies were detected. The third person was a 37-year old former resident of Haiti. She had moved to Belgium 20 days before screening because of assisted reproductive treatment. She also had anti-DENV and anti-chikungunya IgG antibodies, but not IgM.

Predominant signs and symptoms in symptomatic ZIKV cases were rash (93.5%), fever (56.5%), arthralgia (45.7%), myalgia (26.1%), non-purulent conjunctivitis (23.9%) and fatigue (19.6%). One patient had painful paresthesia in the upper and lower limbs, that started in the first week after symptom onset and lasted for 2 months; electrophysiological studies were normal and did not support a diagnosis of Guillain-Barré syndrome.

Among symptomatic travellers, rash (LRP 5.6) and conjunctivitis (LRP 10.8) were independent predictors of ZIKV infection (Table 4). From a pre-test probability Po of 20.3% in symptomatic travellers, the presence of rash and conjunctivitis increased the probability of ZIKV infection to 58.8% and 73.3%, respectively. The Case Definition had a LRP 7.6 and increased the probability to 65.9%.

The Po of ZIKV infection in asymptomatic travellers in our cohort was 1.3%. With 95% certainty, post-test probabilities P1 after negative ELISA results at 15, 20 and 25 days after returning from endemic areas were below 0.3%, 0.1%, and 0.03% (Fig. 1).

Table 4. Clinical presentation of symptomatic travellers, by ZIKV diagnosis (n=227)

Symptoms	ZIKV cases (n) (total n=46)	(%)	Non-ZIKV cases (n) (total n=181)	(%)	LRP#
rash	43	(93.5%)	30	(16.6%)	5.6
fever	26	(56.5%)	98	(54.1%)	1.0
arthralgia	21	(45.7%)	34	(18.8%)	2.4
myalgia	12	(26.1%)	42	(23.2%)	0.3
conjunctivitis	11	(23.9%)	4	(2.2%)	10.8
fatigue	9	(19.6%)	28	(15.5%)	1.3
diarrhea	7	(15.2%)	64	(35.4%)	0.4
respiratory symptoms	3	(6.5%)	25	(13.8%)	0.4
headache	3	(6.5%)	5	(2.8%)	2.4
lymphadenopathy	2	(4.3%)	0		
paraesthesia	1	(2.2%)	0		
gastro-intestinal complaints *	0		7	(3.9%)	
ZIKV Case Definition°	27	(58.7%)	14	(7.7%)	7.6

* *gastro-intestinal complaints included abdominal pain and vomiting*

° *ZIKV Case Definition refers to the European Centers for Disease Control (ECDC) Clinical Case Definition, which defines ZIKV infection by a maculopapular rash with or without fever, and painful joints or muscles or non-purulent conjunctivitis (Case Definition) (15).*

LRP denotes positive likelihood ratio

Discussion

Forty-nine out of 462 travellers (11%) had confirmed ZIKV infection after returning from American territories with active ZIKV transmission. In 2016, ZIKV did not only become the most frequently imported arboviral infection at ITM, but it ranked second only to diarrhea in travel-associated illness. The risk of acquiring ZIKV infection for travellers outside the context of a recognized outbreak will be much lower. Only 3 of 49 travellers (6.1%), all native to Latin America, were asymptomatic. The group of symptomatic and asymptomatic travellers had similar exposure in terms of travel type, destination and duration. The ratio of symptomatic-to-asymptomatic ZIKV infections was greater than 15:1. Given the estimates of this ratio in non-traveller populations in

Micronesia and French Polynesia (1:4.4 and 1:1 (4)(20)), we had anticipated a lower ratio in travellers with similar exposure in our setting. However, several studies have already suggested that asymptomatic ZIKV infections occur in only 0-27% of non-pregnant returning travellers (21–23). In a prospective incidence study in Belgian travellers, only 1 in 9 ZIKV cases (11.1%, 95%-CI [2.0-43]) was asymptomatic (Huits *et al.*, submitted). These observations suggest that, in contrast to the findings from endemic settings, the majority of ZIKV infections in immunocompetent travellers are symptomatic. In pregnant travellers considerably higher rates (25-83%) of asymptomatic ZIKV infection are reported, possibly because of increased immunotolerance and also because the threshold to screen for ZIKV infection is lower (21,24,25). Asymptomatic travellers in our study were younger and had reproductive concerns more frequently. This reflects the awareness of potential hazards of ZIKV infection to pregnancy outcomes that is more relevant in this age-group. Because health-center based cohort studies are less likely to recruit asymptomatic travellers, our findings are subject to selection bias. In addition, the mild disease associated with ZIKV infection may lead to recall bias, leading to an overestimation of rate of asymptomatic ZIKV infection. However, we do believe that our cohort is representative of travellers to ZIKV epidemic territories who are screened for infection in European travel clinics, and that asymptomatic ZIKV infections in non-pregnant travellers from non-endemic countries are less likely to occur than previously assumed.

The clinical presentation of symptomatic ZIKV infection in returning travellers in our cohort was similar to that reported by others (1,26,27). We confirmed that the ECDC Case Definition had a moderate to good positive predictive value for ZIKV infection, because it included the strongest predictors (rash and conjunctivitis) in symptomatic returning travellers during the outbreak.

Viruses that belong to the genus *Flavivirus* (eg. West Nile virus (WNV), dengue virus (DENV), tick-borne encephalitis virus (TBEV), yellow fever virus (YFV) Japanese encephalitis virus (JEV), and ZIKV) express an envelope protein E. The ectodomain of this protein contains 3 highly

immunogenic epitopes (EDI, EDII and EDIII), that are conserved across members of the genus because of their roles in virus attachment, entry and membrane fusion. The antigenic similarity permits categorization of flaviviruses into serocomplexes, but it also leads to antibody cross-reactivity and thus presents a challenge to diagnostic assays (28,29). False positive results of ZIKV-antibody detection assays are common in persons with previous or current heterologous flavivirus infection such as DENV, or after vaccination against flaviviruses (eg. YFV, JEV and TBEV) (30). Analogous to DENV and WNV, ZIKV-infected cells secrete viral Non-Structural protein 1 (NS1). For the Euroimmun ELISA, that uses recombinant ZIKV-NS1 as antibody-binding substrate, lower levels of cross-reactivity and were observed (19). Positive and equivocal ELISA screening results require confirmation by VNT to discriminate between antibodies to ZIKV and to related flaviviruses (28). However, even VNT results should be interpreted with caution, because both acute and convalescent sera of DENV-infected patients may show potent neutralization of ZIKV in vitro (30).

To rule out ZIKV infection as requested by many asymptomatic travellers, it is more relevant to avoid false-negative results. Therefore, testing algorithms with high sensitivity are required. Studies that evaluate commercial anti-ZIKV antibody detection assays tend to address the analytical sensitivity in reference to a comparator, eg. an IgM antibody capture enzyme-linked immunosorbent assay or plaque reduction neutralization test (31,32). For clinical purposes it is important to take the antibody kinetics over time after infection into account, because the duration of detectability of anti-ZIKV IgM (usually present a week after infection) may be short, and IgG may not be present in early samples (29,33). A combined anti-ZIKV IgM/ IgG sensitivity of 100% (95%CI [78.4–100]) was reported for the Euroimmun ELISA at 6-20 DPSO in 17 RT-PCR-confirmed cases (19). An important limitation of our study is that VNT was not performed on samples with negative ELISA results, so our data do not permit us to comment on the sensitivity of ELISA.

While predictive values of tests are affected by pre-test probabilities, likelihood ratios can be applied independently of disease prevalence and maintain their diagnostic value in individual patient care (13). However, as the ZIKV epidemic declines due to waxing herd immunity, the observed frequency of symptoms attributed to ZIKV infection in returning travellers as presented in Table 4 will be reduced. The resulting proportional shifts of presenting symptoms in ZIKV cases vs. non-ZIKV cases will decrease the LRP for these symptoms. This may limit the external validity of our findings of rash, conjunctivitis and indeed, the Case Definition as independent predictors of ZIKV infection outside the outbreak context.

Conversely, the LRN (*i.e.* the percentage of false-negative results divided by the percentage of true- negative results) is independent of the pre-test probability. Its clinical utility relies on the analytical sensitivity and specificity of the assays employed (13). Using the time to seroconversion predicted by Lessler's model (11), our estimates of the LRN for anti-ZIKV ELISA at 20 and 25 days after returning from endemic areas were well below 0.1. Since the pre-test probability at the peak of the outbreak was 11%, a negative ELISA result can be used to confidently rule out a diagnosis of ZIKV infection in returning travellers.

Caution should be taken not to test asymptomatic persons too early. In our retrospective study, 57 of 235 (27.2%) asymptomatic travellers were tested before 20 days, of whom 27 (11.5%) before 15 days post-travel. If greater certainty is desired, ELISA should be repeated after 20 days.

Conclusions

The frequency of ZIKV infection was high in symptomatic travellers who were exposed during the outbreak in the Americas. Rash, conjunctivitis and the Case Definition predicted ZIKV in symptomatic travellers. When asymptomatic, the pre-test probability of ZIKV infection was low. In absence of robust data on anti-ZIKV antibody kinetics and the sensitivity of commercial antibody detection assays from longitudinal studies with a follow-up of ZIKV PCR-confirmed cases, we adopted a practical diagnostic approach that assumed seroconversion to have occurred by 20 days after infection. We consider negative results in a combined ELISA IgM/IgG antibody assay performed at 20-25 days after travel-associated exposure safe to rule out ZIKV infection in clinical practice. Testing for anti-ZIKV antibodies within this timeframe should be considered as an acceptable alternative to deferring conception to minimize the risk of ZIKV congenital syndrome in asymptomatic travellers returning to non-endemic areas.

Fig. 1.

Legend (figure on opposite page)

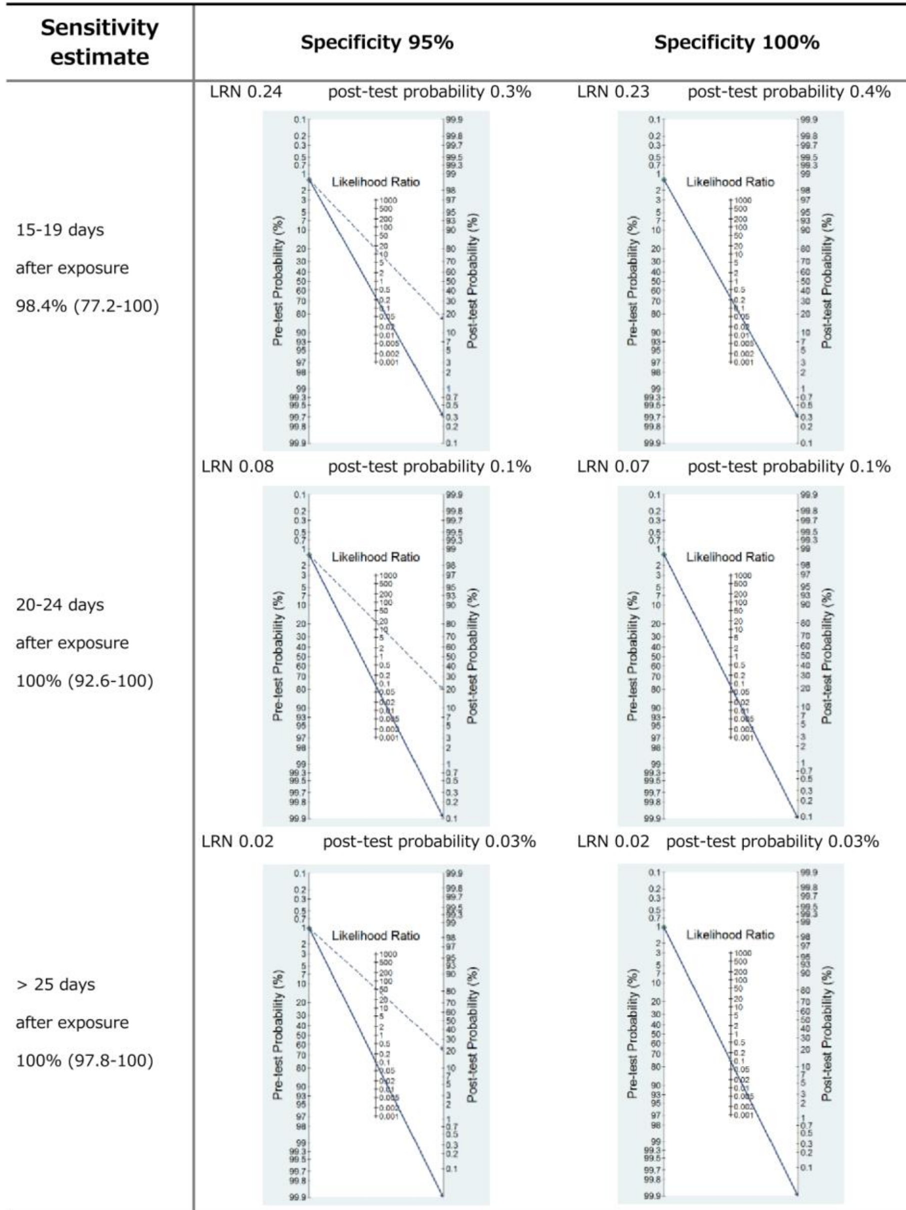
We calculated the Negative Likelihood Ratio (LRN) using the reported specificity of the anti-ZIKV IgM/IgG Enzyme Linked Immunosorbent Assay (ELISA) (Euroimmun, Lübeck, Germany) was 100% (95%-CI [95.9–100.0]) (ref (18)). We calculated LRN for specificities of 95% and 100%.

The sensitivity of anti-Zika antibody detection assays was estimated by adopting lower margins of the 95%-Credible Interval (95%-CrI) for seroconversion in function of the number of days post symptom onset (DPSO) from Lessler et al. and source data (11). Therefore, with 95% certainty, the true post-test probability is lower than the reported values.

The conservative estimates of post-test probabilities of Zika after negative results when tested at 15, 20 and 25 days after travel-associated exposure were calculated and plotted in Fagan nomograms (solid lines). Post-test probabilities of Zika after positive results are plotted only for specificity 95% (dashed lines).

The Fagan nomogram consists of 3 vertical axes: the pre-test probability (left axis), the likelihood ratio (middle axis) and the post-test probability (right axis). (Fagan nomograms were created using Stata 14.2 ("FAGAN: Stata module for Fagan's Bayesian nomogram", Statistical Software Components S457060, Boston College Department of Economics), by Ben Dwamena, 2009.)

Figure 1. Estimated post-test probabilities for negative Zika virus antibody detection results in asymptomatic adults, at prevalence 1.3%



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Chapter 6

Discussion

This thesis focuses on selected challenges in the diagnosis and management of chikungunya (CHIKV) and Zika virus (ZIKV) infections.

Aruba, an island in the Dutch Caribbean, experienced an outbreak of an Asian genotype of CHIKV for the first time from October 2014 to March 2015. In **Chapter 1**, we complemented the diagnostic evaluation of patients during the acute phase of suspected CHIKV infection by an antibody detection assay as performed in Aruba, by molecular investigations performed on the same stored samples from this outbreak at the Institute of Tropical Medicine (ITM) in Antwerp, Belgium. We also assessed the clinical symptoms of CHIKV infection and persistence and predictors of post-chikungunya chronic polyarthralgia (pCHIK-CPA). In **Chapter 2**, we evaluated the diagnostic accuracy of a prototype rapid diagnostic test for CHIKV in our reference laboratory.

The incidence and clinical presentation of ZIKV infection in travellers to areas affected by the ZIKV epidemic in 2016-2017 were studied in **Chapter 3**. We studied the persistence of ZIKV in semen of men with confirmed ZIKV infection at ITM in **Chapter 4**. Finally, we report on the diagnostic approach to ZIKV infection in travellers returning from areas with active vector-borne transmission in **Chapter 5**.

Synopsis of research findings – chikungunya virus

CHIKV infections and post-chikungunya chronic polyarthralgia in Aruba.

As expected, arthralgia, fever and skin rash were the dominant symptoms of CHIKV infection in the acute phase during the 2014-2015 outbreak in Aruba. In **Chapter 1**, we noted that 94% of the interviewed patients with a confirmed diagnosis of CHIKV complained of joint pains. Up to seventy-one percent of patients reported the presence of inflammatory signs (stiffness, edema and redness). All joints were affected, but involvement of knees, ankles, fingers and feet was recorded in more than 40% of instances. pCHIK-CPA lasting longer than 6 weeks was experienced by 44%, and longer than 1 year by 26% of cases.

Comparing the nature, frequency and duration of pCHIK-CPA between outbreaks is difficult because of variation in patient selection, mode and timing of assessment of joint pains, and length of follow-up between reports (1). A systematic review of outbreaks involving ECSA genotype CHIKV in La Réunion and India by Rodriguez-Morales et al., estimated that 40% of patients would develop chronic inflammatory rheumatism (CIR), which was defined as articular rigidity, erythema, edema, and musculoskeletal pain that lasted longer than 2 months after the acute illness (2). In a sub-analysis that included only prospective studies from these outbreaks, a lower prevalence of CIR was reported (25%). This analysis could indicate that retrospective study designs are subject to biases, like selection, recall and reporting bias. Our study design was therefore likely to overestimate the frequency of pCHIK-CPA. Additional limitations in our study were the exclusion of participants younger than 18 years and the potential under-reporting of obesity in our cohort, that may lead to lower and higher prevalence of pCHIK-CPA, respectively. Even so, our observations on pCHIK-CPA are in line with prospective data from Asian genotype CHIKV outbreaks in Colombia (arthralgia persisting in 44.3% of infected patients at 21 weeks), and Suriname (arthralgia persisting in 22.2% of viremic patients at 6 months) (3,4).

On the diagnosis of CHIKV infections in the acute phase.

During the outbreak in Aruba, the diagnosis of acute CHIKV infections was frequently missed because diagnostic facilities were limited to antibody detection assays alone. By 6 days after symptom onset, CHIKV-specific IgM antibodies were detectable by immunofluorescent assay in only 80% of cases. CHIKV-RNA remained detectable by RT-PCR in approximately half of the patients until the end of the sampling timeframe in our study, *i.e.* 10 days post symptom onset. Earlier studies, that were performed during ECSA genotype CHIKV outbreaks and that used fewer serum samples per day after symptom onset, described 100% detectability of IgM by the end of the first week after symptom onset (5–7). By that time, only a minority of patients were found to be viremic. The Centers for Disease Control and Prevention therefore adopted a testing algorithm for the diagnosis of CHIKV infection that does not include nucleic acid detection assays in sera collected 6 days or more after symptom onset (8).

Predictors of post-chikungunya chronic polyarthralgia

We found three independent risk factors for pCHIK-CPA. Female gender (odds ratio 5.9 [2.1-19.6]) and the pattern of joint involvement (odds ratio 7.4 [2.7-23.3]) predicted chronic sequelae of CHIKV infection, as was frequently reported following outbreaks by different genotypes and in various geographical settings across continents (4,9–12). They are discussed in greater detail in Chapter 1. A novel predictor of pCHIK-CPA that we identified is the persistence of viremia beyond 7 days of symptom onset (odds ratio 6.4 [1.4-34.1]). As explained in the preceding paragraph, viral RNA was frequently detected up to 10 days after symptom onset in patients infected with an Asian genotype CHIKV in Aruba. Future studies should determine if viral persistence in Asian-Caribbean lineage CHIKV infections is prolonged compared to infections with other epidemic strains. Experimental data from murine and non-human primate models suggest that the Asian-Caribbean lineage CHIKV is less virulent and less arthritogenic than other CHIKV genotypes (WA, ECSA) (13,14). Prolonged viremia in the host could then be attributed to reduced immunogenicity of the infecting CHIKV strain. Host immunogenetic differences in the study population as an alternative explanation for delayed clearance of CHIKV, are less likely because the Aruban population is of mixed descent and origin.

A rapid diagnostic test for CHIKV infections ?

The clinical importance of confirming CHIKV diagnosis was highlighted by the considerable burden of chronic morbidity caused by this infection. Assays detecting virus-specific antigen in the acute stages of infection could significantly enhance the diagnostic capacity for CHIKV infections, as has been demonstrated for dengue virus infections (15). In Chapter 2, we found that a prototype immunochromatographic test that used mouse monoclonal antibodies (MAbs) to capture the CHIKV E1-antigen had fair diagnostic sensitivity for ECSA genotype CHIKV but low sensitivity for Asian-Caribbean genotype from Aruba, and poor overall specificity. The remarkable difference in sensitivity was explained by the low affinity of MK47 MAbs for the CHIKV envelope protein of strains

that carried a single amino acid substitution (D350E in the 6K-E1 protein) (16). While the performance of the test under study was disappointing, we gained valuable insight into the consequences of phenotypic variability of CHIKV. Antibodies that react across genotypes will be required for the development of a rapid test for CHIKV. However, the use of broad-reacting antibodies is likely to result in lower specificity, due to cross-reactivity with other alphaviruses or with unrelated pathogens (17,18).

Synopsis of research findings – Zika virus

The incidence of ZIKV infection in travellers to the Americas in 2016

In **Chapter 3**, we investigated the ZIKV incidence rate among travellers from Belgium to epidemic territories. The coincidence of participant recruitment in a observational study of febrile illness during travel with the ZIKV outbreak in the Americas, provided us with a unique opportunity to prospectively study the incidence of ZIKV infection in adult travellers. In this population, we observed a ZIKV incidence rate of 17.0% (95%CI [7.8 - 32.2]) per month of travel. The risk of acquiring ZIKV infection outranked all other travel-associated health hazards during the 2016 outbreak except for traveller's diarrhea, which affects up to 20% of international travellers during a 2-week stay (19). The incidence rate of ZIKV infection in travellers to endemic areas also exceeded historical assessments of dengue virus incidence rates among international travellers, which were estimated to range from 2.2 to 59 per 1,000 person-months in seroconversion studies (20–25). According to surveillance data, approximately 8000 ZIKV cases were imported into the USA and the EU (26). The high volume of international travellers to the Americas during the ZIKV outbreak and the high attack rates combined, suggest that these surveillance data really underestimate the true number of cases imported into non-endemic areas. This illustrates the frequently issued cautionary advice to rely on surveillance reports (which lack denominator data) for the risk analysis of travel-associated infections (27,28). The high attack rates in a susceptible population

would be compatible with the rapid dispersal of Zika virus during outbreaks, as witnessed across Latin America and the Caribbean (29).

A particular strength of our study is the use of RT-PCR in urine and on capillary blood blotted onto filter paper during illness by the travellers, and virus neutralization testing (VNT) in addition to seroconversion, to diagnose and confirm ZIKV infection in our cohort. Important limitations limit the generalizability of our findings. First, our sample size remained relatively small because of the declining epidemic towards the end of 2016. Second, our analysis by travel destination was a simplification of the risk factors that determine the risk of acquiring vector-borne disease during travel (transmission intensity, geographic and climatological conditions, population density, activities, etc.)). Instances of ZIKV infection per travel destination in our cohort correlated with ongoing local transmission, as reported by national health authorities of these countries to the Pan American Health Organization (PAHO).

The high incidence of ZIKV infection in travellers is likely to reflect the high intensity of vector-borne transmission of this newly emerging infection during the epidemic.

Clinical presentation of Zika virus infection in travellers

Our studies confirmed that symptomatic ZIKV infection in non-pregnant travellers produced a mild flu-like illness that is almost always associated with rash (**Chapters 3, 4** and **5**). We did not observe any severe neurological complications, and none of our subjects were hospitalized. The main presenting symptom, affecting more than 90% of cases in the prospective and cross-sectional studies was exanthema ('rash'). This is in line with other published records of the clinical presentation of ZIKV infection in travellers (28,30). However, fever and non-purulent conjunctivitis were observed less frequently in our series (up to 56% and 24% of cases, respectively). We estimated that the ECDC Clinical Case Definition for identifying cases among ill travellers returning from areas with an ongoing ZIKV outbreak served its purpose rather well (31). It had a positive likelihood ratio of 7.6, which indicates a good confirming power of the Case Definition for clinicians.

Our most significant contribution to the clinical assessment of ZIKV infection in travellers is the finding that asymptomatic infections in non-pregnant travellers from non-endemic areas occur much less frequently (10%) than previous studies from endemic areas indicated (reported at 80%). In **Chapter 3**, only 11% of ZIKV cases was asymptomatic. Although the sample size was small (n=49), the prospective design of this observational study eliminated the potential for selection bias that would result from increased health seeking behavior by symptomatic persons. In our cross-sectional analysis of subjects with similar travel-associated exposure (see **Chapter 5**), ZIKV was diagnosed in 20% of symptomatic, but only in 1.3% of asymptomatic returning travellers. One hypothesis to explain the lower frequency of asymptomatic ZIKV infection in travellers compared to people living in endemic areas, is reduced previous exposure to other flaviviruses. A recent systematic review of the available published data also arrived at a low prevalence of asymptomatic ZIKV infection in the subgroup of returned travellers (0.02, 95%-CI [0.00–0.31]) (32).

We did not recruit any pregnant women in our studies. Pregnant women are more vulnerable to ZIKV infection than non-pregnant women or adult men (33). ZIKV RNA detection in blood, the female genital tract, placenta and fetal tissues may be prolonged in pregnancy (34,35). The humoral immune response to ZIKV infection during pregnancy may be blunted and result in reduced seroconversion rates, as was demonstrated for the response to yellow fever vaccination (36). In contrast to other travellers, ZIKV infection was often found to be asymptomatic in pregnancy (55%, 95%-CI [33–78]) (32). And finally, congenital Zika syndrome occurred at similar rates in pregnancies after symptomatic or asymptomatic infection (37–39). Our findings of low pre-test probabilities of ZIKV infection in asymptomatic returning travellers can therefore not be applied to pregnant women.

Zika virus in semen and the male reproductive tract

In **Chapter 4**, we investigated the potential for sexual transmission of ZIKV infection by studying the frequency and persistence of ZIKV in semen of returning travellers. Sixty percent of men with confirmed, symptomatic ZIKV infection had detectable ZIKV RNA in their semen for a median duration of 3 months. A positive RT-PCR result in semen indicates the presence of viral RNA, rather than viable virions. It is important to note that we only succeeded in isolation of ZIKV from one semen sample with a high viral load, that was collected at 11 days post symptom onset. Although the longest documented interval between systemic symptoms of ZIKV infection in the index case and sexual transmission to his female partner is 44 days, we would hesitate to rule out the presence of infectious ZIKV based on failure to isolate the virus, as long as viral RNA is detected in semen.

Our study represents the only European prospective follow-up on ZIKV persistence in semen in a cohort of returning travellers. In spite of the small sample size, our observations of the frequency of detectable Zika virus RNA in the semen of infected men were corroborated by larger cohort studies (40,41). These cited studies found a similar decline of ZIKV RNA shedding (to less than 11%) during the 3 months after symptom onset (40,41).

Even when ZIKV RNA was not detected in semen, we observed signs of tissue damage to the male reproductive tract in the majority of infected men. Semen samples of eleven men were available for microscopic analysis. White blood cells were present in eleven and erythrocytes in nine semen samples. After exclusion of a vasectomized case, oligospermia was detected in six of 10 samples. Although leucospermia, hemospermia and oligospermia have not been reported consistently in infected humans, studies in animal models have demonstrated that ZIKV infection can lead to structural damage in the testes (42). To date, only one other prospective study documented the effect of ZIKV infection sperm counts in humans (43). In this study, Joguet *et al.* demonstrated that ZIKV infection resulted in harmful effects on markers of male reproduction, such as total sperm count, morphology, motility and concentrations of reproductive hormones, during 120 days of follow-up.

Ruling Zika virus in ⋯ or out

As was demonstrated for the diagnosis of CHIKV in Chapter 1 of this thesis, a thorough understanding of the viral kinetics and antibody response to infection is essential to determine diagnostic strategies. At the start of the ZIKV outbreak in the Americas, important details in this respect were missing. Knowledge gaps included the natural history of infection, viral kinetics and persistence in different body fluids, and the onset and duration of the human antibody response to the epidemic Asian lineage ZIKV strains in specific host populations (44–46). Only a few diagnostic assays had been developed, and these had not been standardized for the detection of ZIKV (47,48). Because symptomatic ZIKV infection shares signs and symptoms with many other infections (e.g. dengue and chikungunya, HIV seroconversion, measles, scarlet fever, rickettsial infection, leptospirosis, parvovirus, enterovirus, rubella, and secondary syphilis), laboratory diagnosis is required for confirmation of ZIKV infection (49).

Recognizing these limitations, in **Chapter 5** we evaluated our diagnostic approach to confirm or rule out a diagnosis of ZIKV infection in symptomatic and asymptomatic travellers returning from the Americas during the epidemic. In persons evaluated for ZIKV infection at ITM during this epidemic period, we found a high prevalence (20.3%) of cases among symptomatic travellers, but a low prevalence (1.3%) among asymptomatic travellers who had comparable travel duration and destinations. For couples who wish to conceive and anticipate a risk of congenital ZIKV syndrome after travel-associated exposure to ZIKV infection, concerns over false negative test results prevailed concerns over false positive test results. Another important finding is that the post-test probability of ZIKV infection after a single-sample negative result in a combined IgM/IgG antibody ELISA at 20-25 days post-travel is well below 0.1%.

A major strength of our study is the performance of ZIKV-specific ELISA in clinically relevant and well documented timeframes after symptom onset or travel-associated exposure. All cases identified by positive ELISA results were confirmed by VNT, which indicated excellent specificity of the ELISA. An important limitation to our work is the lack

of a comparator to confirm the diagnostic sensitivity of the combined IgM/IgG ELISA. This was discussed in greater detail in **Chapter 5**.

Implications for patient care, public health and future research - chikungunya virus

We describe a significant burden of CHIKV-attributable morbidity during and after an outbreak of this *alphavirus* in Aruba. Obtaining a specific diagnosis for this infection is relevant to the management of patients who may suffer chronic sequelae of the infection, but it is also important for outbreak alerts and disease surveillance by health authorities in regions where multiple arboviruses co-circulate. Our finding that viremia was prolonged in half of the patients infected with an Asian-Caribbean lineage CHIKV has increased the window of opportunity for the detection of viral RNA to at least 10 days after symptom onset. Based on this finding and when available, diagnostic algorithms should adopt CHIKV-specific RT-PCR testing in serum to 10 days post symptom onset when infection with an Asian genotype is suspected.

The identification of prolonged viremia as an independent risk factor for pCHIK-CPA (in addition to previously recognized predictors such as female gender and pattern of joint involvement), provides a rationale for performing CHIKV-specific RT-PCR beyond 7 days and up to 10 days after symptom onset. Whether this predictor could be used in clinical practice to determine the prognosis for development of pCHIK-CPA requires further prospective evaluation. The prediction of patients at risk for pCHIK-CPA in the acute phase of CHIKV infection permits efficient monitoring and tailored management of those patients (50). It would also enable cost-effective recruitment of patients in prospective studies of experimental therapeutic interventions to reduce the complications of CHIKV infection (51–53).

A rapid diagnostic test for CHIKV that meets the ASSURED criteria (Affordable, Sensitive, Specific, User-friendly, Robust, Equipment-free and Deliverable to those who need them) is still not available (54). The high degree of genetic variability that is characteristic for many RNA

viruses, affected the affinity of MAbs-binding to E1-antigens between CHIKV lineages. The diagnostic accuracy of new immunochromatographic tests for CHIKV should be evaluated against different genotypes and lineages, and these tests should be validated in field settings during outbreaks. At ITM, we will remain involved in the evaluation of a next generation of rapid immunochromatographic or molecular tests for CHIKV.

The intricacies of genomic variation between Asian-Caribbean and other CHIKV lineages and the host immune response may also hold a key to observed differences in the clinical presentation and occurrence of long-term sequelae of CHIKV infection between individuals and outbreaks. The study of this potential association will be facilitated by recent advances in next generation sequencing technology and is the subject of future research collaborations (55,56).

Implications for patient care, public health and future research - Zika virus

The evidence to provide a well-informed pre-travel advice to travellers with destinations affected by the ZIKV outbreak in 2016 on the risk of acquiring the infection was not available (57,58). Retrospectively, the high attack rate of ZIKV in travellers from Belgium, a non-endemic country, justify the precautionary measures and travel restrictions that were recommended by health practitioners, national and international authorities to persons at risk of complications of ZIKV infection (28,59).

The most important clinical sign of ZIKV infection in travellers was rash, rather than fever. Although the ECDC Clinical Case Definition for the identification of suspected ZIKV cases performed reasonably well, we expect the clinical utility of the Case Definition to decrease outside the context of epidemic exposure to ZIKV.

The prevalence of asymptomatic ZIKV circulation in humans and in non-human primates is a driver for propagation of outbreaks in endemic

areas. It is therefore relevant to surveillance efforts by public health authorities. Contrary to earlier assumptions, asymptomatic ZIKV in non-pregnant travellers is rare. It is therefore unlikely that introduction of ZIKV by asymptomatic viremic travellers will cause localized outbreaks in non-endemic countries where a suitable vector is present. For clinical purposes, we recommend that physicians only request laboratory confirmation of ZIKV infection in travellers who present with symptoms, with the exception of asymptomatic pregnant couples or those who wish to conceive on a short notice.

The frequent detection of viral RNA in semen of symptomatic men with confirmed ZIKV infection was associated with a potential for sexual transmission of this *flavivirus* (60). Although this route of transmission will not sustain ZIKV circulation in non-endemic areas, the finding itself was distressing to patients. The spontaneous clearance of viral shedding in semen over a median of 3 months was a relief to them. Our study (**Chapter 4**) contributed to the evidence base for the 2018 update of the CDC recommendations for 'Preconception Counseling and Prevention of Sexual Transmission of Zika Virus'. These now state that when a couple or the male partner has traveled to areas with risk for ZIKV infection and the couple plans to conceive, the period to use condoms or abstain from sex can be reduced from 6 to 3 months after the male partner's symptom onset or last possible ZIKV exposure (if asymptomatic) (61,62). However, in order to prevent sexual transmission of ZIKV we also provided evidence that would permit a further reduction of this period to less than one month after travel to endemic areas, by obtaining a negative result for ZIKV-antibody detection in a single serum sample at 20 to 25 days after travel-associated exposure. After a positive test result, the physician could offer additional ZIKV-specific RT-PCR testing on the man's semen if a wish to conceive at short term exists, or in the case of active assisted reproductive treatment. Failure to detect ZIKV-RNA in two consecutive semen samples makes infection of semen, and therefore sexual transmission of ZIKV, very unlikely.

In March 2017, I was invited to a meeting of experts convened by WHO in Geneva, Switzerland, to review existing evidence on the sexual transmission of ZIKV and to identify research gaps (63,64). The scientific community has managed to address some of these gaps, but important questions remain. Examples are: 'Do genomic differences between infecting ZIKV lineages lead to differential compartmentalization or increased gonadotropism?'; 'Which are the target cells for ZIKV entry?'; 'Does the route of transmission (via mosquito or via vaginal intercourse) pose a differential risk for the fetus and for neonatal outcomes to mothers who were infected during pregnancy?'; 'Does infection with ZIKV result in lifelong immunity and protection of the fetus?'; 'What are the long-term effects of ZIKV infection on male fertility in humans?'. One of the research questions that we are currently focusing on in relation to prolonged ZIKV-RNA detection in semen is: 'Is this virus infectious?' Isolation of viruses is widely regarded as the only direct and definitive approach for proving infectivity (65). Disappointed by the low success rate of ZIKV isolation from semen in our experience, we have investigated alternative approaches to detect its replication competence. We found viral protein in semen with high viral loads using a prototype ZIKV NS1 antigen test (66). We are currently exploring strategies to target anti-sense ZIKV-RNA in semen by RT-PCR which, when present, would also indicate ongoing viral replication. However, as with attempted virus isolation, negative results are unlikely to exclude the potential for sexual transmission.

Concluding remarks

This thesis described clinically relevant aspects in the diagnosis and management of two arbovirus infections of global importance that have recently emerged as major epidemics, CHIKV and ZIKV. Media coverage of these *Aedes*-borne infections may have given the impression that the emergence of pandemics like these are isolated events. They are not. We share this world with each other, with animals and livestock, plants, insects and microbes. For all its benefits, through population growth, global trading and tourism, air travel, disruption of ecosystems by urbanization, agricultural intensification and climate change, our

'interconnected global community' also facilitates the exchange and rapid dissemination of pathogens (67). The rate at which new infectious disease emerge is increasing (68). Sixty percent of the newly emerging pathogens, and almost all that caused the recent pandemics (e.g., influenza, Ebola, MERS, and the *Aedes*-borne infections chikungunya and Zika) are zoonotic in origin (69–72). The sustained transmission of arboviruses usually does not require infection of humans, because they are dead-end hosts (73). However, the adaptation of arboviruses to anthropophilic vector species will result in the establishment of urban epidemic transmission cycles. This has happened for dengue virus, CHIKV and ZIKV, and it is waiting to happen for many other arboviruses, like Mayaro, Oropouche and Venezuelan equine encephalitis viruses (74).

The study of arboviruses and arboviral disease demands recognition of the interrelated nature of human and animal health. I would like to make a strong case for intensifying interdisciplinary cross-talk and collaboration with other 'Blind Men' to describe 'the Elephant'. The adoption of an integrated approach to individual, population, and ecosystem health as developed by the 'One Health Initiative' should strengthen our preparedness for the management of arbovirus infections at public health and individual level (75).

From a clinician's point of view, we need to be able to diagnose newly emerging arbovirus infections and to manage unexpected complications. To design new tools for prevention, diagnosis and management of arboviral infections, much more research is needed. Ideally, this research should take place at the interface of the basic sciences, clinical medicine and the community.

For me personally, the work on chikungunya and Zika virus infections presented in this thesis has been an fascinating initiation in the field of arbovirus research.

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Summary

Arthropod-borne viruses (arboviruses) are viruses that can be transmitted between animal or human hosts by arthropod vectors, such as mosquitoes, ticks and sandflies. Transmission of virus occurs when the arthropod bites the host to feed on his blood. An infectious bite may then lead to a wide range of symptoms, from inapparent (asymptomatic) to severe disease and death. Approximately 150 virus species are known to cause human disease, and some of them have threatened human health for centuries. Others, like chikungunya virus (CHIKV) and Zika virus (ZIKV), have caused outbreaks on a global scale over the past decades. CHIKV and ZIKV belong to different families of viruses (*Togaviridae* and *Flaviviridae*, respectively), but they are both primarily transmitted by *Aedes* species mosquitoes, that are widely distributed across the continents. The emergence of CHIKV and ZIKV into new territories has emphasized that significant gaps exist in our knowledge of the natural history and clinical epidemiology of arboviral illnesses.

As explained in the **Introduction**, these gaps present many challenges to the diagnosis and management of CHIKV and ZIKV infections. People who are infected or at risk of infection, clinicians, public health authorities and policy makers could benefit from multidisciplinary scientific efforts to advance our understanding of the risk factors, clinical presentation and diagnosis of *Aedes*-borne infections. The studies that were conducted in the context of this PhD thesis aim to contribute to these efforts.

CHIKV was first introduced in the Caribbean and the New World in December 2013. It spread rapidly, and from October 2014 to March 2015, the outbreak affected the island Aruba in the Dutch Caribbean. The diagnostic capacity for CHIKV infections on the island at that time was limited to CHIKV-specific antibody detection by an immunofluorescence assay at the Landslaboratorium Aruba. At the Institute of Tropical Medicine in Antwerp (ITM), we retrospectively tested the sera of 498 patients who were referred to the Landslaboratorium for CHIKV diagnostics by their primary care physicians during the outbreak (**Chapter 1**). Two-hundred sixty-nine CHIKV cases were identified, 210 by antibody detection and in addition 59 (28%) cases using real-time reverse transcription polymerase chain

reaction (RT-PCR). This finding highlights the substantial risk of underdiagnosis in field settings where RT-PCR is not available and follow-up samples are not easily obtained. Hundred seventy-one of 248 patients who were eligible for interview were contacted by telephone. We found arthralgia, fever and skin rash to be the dominant acute phase symptoms. Twenty-six percent of cases suffered from persisting joint pains that lasted longer than one year. Persistence of joint pains was predicted by female gender of the patient (odds ratio 5.9, 95% confidence interval (CI) [2.1-19.6]), the pattern and number of joints involved in the acute phase of infection (odds ratio 7.4, 95% CI [2.7-23.3]), and viremia beyond 7 days of symptom onset (odds ratio 6.4, 95% CI [1.4-34.1]).

The considerable burden of long-lasting sequelae of CHIKV infection and the poor performance of antibody detection-based assays in the acute phase, emphasize the need for improved diagnostics. In **Chapter 2**, we evaluated a prototype immunochromatographic rapid diagnostic test that uses mouse antibodies to target the envelope protein E1 of CHIKV. When evaluated in a panel of clinical samples from returning travellers that contained ECSA genotype CHIKV (different lineages), the test had fair diagnostic sensitivity (88.9%, 95% CI [56.5 - 98.0]), but the sensitivity for samples from Aruba, containing Asian genotype CHIKV (33.3%, 95% CI [19.2-51.2]) was low. The overall specificity (83.1%, 95% CI [71.5-90.5]) of the test against sera from patients with other febrile conditions or sera that contained other alphaviruses or flaviviruses was poor. We suggest further development of a rapid test for CHIKV requires the use of antibodies that react across CHIKV genotypes and that the performance of such a new assay should be evaluated against different CHIKV genotypes.

ZIKV, a member of the family *Flaviviridae*, was considered a cause of mild illness or, in 80% of cases, asymptomatic infection. However, its emergence in French Polynesia (2013) and the Americas (2015) has unveiled associations of ZIKV infection with neurological disease and, when pregnant women are infected, with microcephaly and other birth defects. In addition, the notion that this arbovirus can be transmitted from person to person by sexual intercourse, has caused great concern

among scientists, health professionals and the general public. Precautionary advice was given to travellers from non-endemic areas, who consulted travel clinics for risk assessment prior to a journey into areas affected by the outbreak. This advice included travel restrictions for pregnant women. Upon return after travel, clinicians and laboratories were overwhelmed by the demand for diagnostic evaluations for ZIKV. The evidence base for diagnosis and management of ZIKV infection, particularly in regard to family planning, was small.

In a prospective cohort study among 55 adult participants who traveled to areas with epidemic vector-borne transmission in the Americas in 2016 (**Chapter 3**), we observed 9 cases of ZIKV infection. The ZIKV incidence rate was 17.0% (95% CI [7.8 – 32.2]) per month of travel, and during the outbreak it ranked second only to travellers' diarrhea among travel-associated health hazards. Symptomatic infection presented as an exanthematous, rather than a febrile illness. Only one of 9 ZIKV-cases (11.1%) was asymptomatic, suggesting that asymptomatic ZIKV infection in travellers is much lower than previously reported in studies from endemic areas.

The majority of reports of sexual transmission of ZIKV involved male-to-female sexual intercourse. The virus had been detected in high loads, and it was isolated from semen samples for up to 69 days after the onset of symptoms. The potential for sexual transmission of ZIKV seems therefore closely associated to viral persistence in semen, which we studied in symptomatic returning travellers with confirmed ZIKV infection (**Chapter 4**). ZIKV RNA was detected by RT-PCR in the semen of nine out of 15 participants (60%). It remained detectable for 83 days after symptom onset (95% CI [57–108]), and the longest duration of viral shedding in semen recorded in our cohort was 144 days after symptom onset. ZIKV was successfully isolated from one sample only, but as long as viral RNA can be detected in semen, we would not exclude the potential for sexual transmission of ZIKV. In the semen samples of 11 participants whose semen was microscopically analyzed, we found leukocytes (n=11), red blood cells (n=10) and oligospermia (n=6). These abnormalities occurred irrespective of Zika virus detection in semen and may indicate some degree of tissue damage to the male reproductive tract.

At the start of the outbreak in the Americas, validated diagnostic assays for ZIKV infection were in scarce supply. At ITM, symptomatic travellers were tested with a ZIKV-specific RT-PCR on serum samples upon presentation within 7 days post symptom onset (DPSO) and on urine within 14 DPSO and an anti-ZIKV Immunoglobulin (Ig)M and IgG Enzyme Linked Immunosorbent Assay (ELISA). All positive or equivocal anti-ZIKV IgM/IgG results were considered diagnostic only when confirmed by ZIKV virus neutralization testing (VNT). Asymptomatic travellers were tested using ELISA only, preferably from 20 days after the last exposure. In **Chapter 5** we performed a cross-sectional cohort analysis to evaluate our approach to the diagnosis of Zika infection in non-pregnant travellers. During a 12 month period, we evaluated 462 travellers. ZIKV infection was confirmed in 49, and was frequent in symptomatic cases (46/227, 20.3%), but not in asymptomatic persons (3/235, 1.3%). Asymptomatic travellers had similar baseline characteristics, but were younger (median age 31 vs. 37 years, p=0.01) and had reproductive concerns more often (75.8% vs. 24.2%). Rash (positive likelihood ratio (LRP) 5.6) and conjunctivitis (LRP 10.8) predicted ZIKV infection. The post-test probability of a negative ELISA result at 20-25 days was below 0.1%. We consider negative ELISA results at 20-25 days after exposure a safe strategy to rule out ZIKV infection. Testing for ZIKV-specific antibodies within this timeframe could be particularly valuable in the management of returning travellers who wish to conceive.

Samenvatting

(Nederlands)

Arbovirussen ('arthropod-borne virussen') zijn virussen die overgedragen worden tussen gewervelde dieren of mensen enerzijds, en geleedpotige vectoren zoals muggen en teken anderzijds. De overdracht van het virus vindt plaats wanneer de geleedpotige de gastheer bijt om zich met diens bloed te voeden. Een beet van een geïnfecteerde vector kan leiden tot infectie met het virus die zeer wisselend kan verlopen, variërend van asymptomatisch tot ernstige ziekte en overlijden. Het aantal arbovirus infecties dat ziekte bij de mens veroorzaakt wordt geschat op 150, en enkele daarvan vormen al eeuwen een plaag. Anderen, zoals chikungunya virus (CHIKV) en Zika virus (ZIKV), veroorzaakten in de laatste decennia wereldwijd grote epidemieën. CHIKV en ZIKV behoren tot verschillende virusfamilies, respectievelijk de families *Togaviridae* en *Flaviviridae*), maar zij delen dezelfde vector. Dit zijn steekmuggen van het geslacht *Aedes*, dat wijd verspreid is op alle continenten. De opkomst van CHIKV en ZIKV en geografische uitbreiding naar nieuwe gebieden heeft belangrijke lacunes blootgelegd in onze kennis van het natuurlijk beloop en de klinische epidemiologie van deze arbovirus infecties.

Zoals ik toelicht in de **Introductie**, vormen deze lacunes grote uitdagingen voor de diagnose en het beleid bij CHIKV en ZIKV infecties. Multidisciplinair wetenschappelijk onderzoek dat tot een beter begrip van de voorspellers en de klinische presentatie van deze '*Aedes*-borne' infecties en tot verbetering van de diagnostische mogelijkheden leidt, is van belang voor mensen met (risico op) CHIKV of ZIKV infecties, voor clinici, voor gezondheidsautoriteiten en voor beleidsmakers. Het doel van mijn studies in het kader van dit doctoraat is om een bijdrage te leveren aan deze kennisontwikkeling.

De eerste uitbraak van CHIKV in het Caraïbisch gebied en de Nieuwe Wereld was in December 2013. De uitbraak verspreidde zich snel, en bereikte het eiland Aruba in het Nederlands Caraïbisch gebied in oktober 2014. De diagnostiek voor CHIKV infecties op het eiland was op dat moment beperkt tot een immunofluorescentie test voor de detectie van CHIKV-specifieke antilichamen, uitgevoerd door het Landslaboratorium Aruba. Op het Instituut voor Tropische Geneeskunde in Antwerpen (ITG), hebben wij retrospectief de sera van

498 patiënten getest, die tijdens de uitbraak (van oktober 2014 tot maart 2015) door hun huisarts naar het Landslaboratorium verwezen waren voor CHIKV diagnostiek (**Hoofdstuk 1**). Wij identificeerden 269 gevallen van CHIKV , 210 door het aantonen van CHIKV-specifieke antilichamen en 59 (28%) extra gevallen door detectie van CHIKV RNA middels 'real-time reverse transcription polymerase chain reaction' (RT-PCR). Deze bevinding toont aan dat de diagnose vaak gemist wordt in laboratoria waar deze technologie (RT-PCR) niet voor handen is, en waar het verkrijgen van opvolgstalen niet vanzelfsprekend is. Honderd een-en-zeventig van 248 patiënten van 18 jaar of ouder met een bevestigde CHIKV infectie die in aanmerking kwamen voor een interview waren werden telefonisch gecontacteerd. De belangrijkste symptomen tijdens de acute CHIKV infectie waren gewrichtsklachten, koorts en huiduitslag. Zes-en-twintig procent van de patiënten met gewrichtspijnen rapporteerde aanhoudende pijn voor de duur van een jaar of langer. Voorspellers voor deze chronische gewrichtspijnen waren vrouwelijk geslacht van de patiënt (odds ratio 5.9, 95%-betrouwbaarheidsinterval [2.1-19.6]), het aantal en patroon van de aangedane gewrichten in de acute fase van infectie (odds ratio 7.4, 95%-betrouwbaarheidsinterval [2.7-23.3]) en, voor het eerst gedocumenteerd in dit proefschrift, aanwezigheid van CHIKV in het bloed (viremie) die langer aanhield dan 7 dagen na start van de symptomen (odds ratio 6.4, 95%-betrouwbaarheidsinterval [1.4-34.1]).

De aanzienlijke chronische ziektelast van CHIKV infecties en het matige vermogen van antilichaam-detectie testen in de acute fase, benadrukken de noodzaak voor betere diagnostiek in die acute fase van de ziekte. In **Hoofdstuk 2**, onderzochten wij een prototype immuunchromatografische diagnostische sneltest die muis antilichamen gebruikt als substraat voor het E1-eiwit van de CHIKV enveloppe. Bij evaluatie van de test in een panel van klinische stalen dat Eastern/Central/Southern African (ECSA) genotype CHIKV (met verschillende lijnen van verwantschap) bevatte en die verkregen werden van teruggekeerde reizigers die in het ITG gediagnosticeerd werden, vonden wij een redelijke diagnostische sensitiviteit (88.9%, 95%-betrouwbaarheidsinterval [56.5 - 98.0]), maar de sensitiviteit voor stalen uit Aruba met daarin uitsluitend Aziatisch genotype CHIKV was laag

(33.3%, 95%-betrouwbaarheidsinterval [19.2-51.2]). De specificiteit (83.1%, 95%-betrouwbaarheidsinterval [71.5-90.5]) van de sneltest in sera van patiënten met overige koortsende ziekten of sera met daarin andere alphavirussen of flavivirussen, was matig. Voor de verdere ontwikkeling van een sneltest voor CHIKV deden wij de aanbeveling om antilichamen te gebruiken die reageren met alle CHIKV genotypen. De diagnostische nauwkeurigheid van een dergelijke nieuwe test dient vervolgens geëvalueerd te worden tegen genotypen afkomstig van verschillende CHIKV uitbraken.

ZIKV, een virus van de familie *Flaviviridae*, werd aanvankelijk slechts gezien als de oorzaak van milde ziekteverschijnselen in 20% van de geïnfecteerde personen. Echter, tijdens de opkomst van ZIKV in Frans Polynesië (2013) en in het Amerikaanse continent (2015) bleek ZIKV infectie geassocieerd te zijn met neurologische aandoeningen en, bij infectie in de zwangerschap, met foetale microcefalie en andere geboorte-afwijkingen. Bovendien veroorzaakte de bevinding dat dit arbovirus ook via sexueel contact van mens tot mens kon worden overgedragen veel commotie onder wetenschappers, gezondheidswerkers en het brede publiek. Aan reizigers uit niet-endemische gebieden die voor reisadvies kwamen werd geadviseerd voorzorgsmaatregelen te treffen wanneer zij naar gebieden afreisden die getroffen waren door de ZIKV epidemie. Deze maatregelen omvatten onder meer een negatief reisadvies voor zwangere vrouwen of voor mensen met een zwangerschapswens. Na terugkeer van een reis uit de aangedane gebieden werden artsen en laboratoria overspoeld door de vraag naar diagnostiek voor ZIKV infecties. Op dat moment schoot het best beschikbare bewijs tekort voor goede diagnostiek en een gefundeerd beleid bij de verdenking op ZIKV infectie, met name waar het een eventuele zwangerschapswens betrof.

In een prospectieve cohort studie onder 55 volwassen deelnemers die naar gebieden met epidemische transmissie van ZIKV in Latijns Amerika en de Caraïben afreisden in 2016, observeerden wij 9 gevallen van ZIKV infectie (**Hoofdstuk 3**). De incidentie bedroeg 17.0% (95%-betrouwbaarheidsinterval [7.8-32.2]) per maand op reis, waarmee ZIKV infectie na reizigersdiarree het grootste gezondheidsrisico voor reigers

was tijdens de uitbraak. Symptomatische ZIKV infectie presenteerde zich vaker met huiduitslag dan met koorts. Tot slot vonden wij in deze studie dat slechts 1 van de 9 patiënten (11.1%) asymptomatisch was, hetgeen erop duidt dat asymptomatische ZIKV infectie in reizigers veel minder vaak voorkomt dan in studies uit endemische gebieden werd gerapporteerd.

De meeste rapporten over geslachtelijke overdracht van ZIKV betroffen de seksuele overdracht van man op vrouw. Het virus werd in hoge concentraties aangetroffen in sperma en geïsoleerd uit spermastalen tot 69 dagen na aanvang van systemische symptomen. Omdat het potentieel voor seksuele overdracht van ZIKV nauw verbonden leek met de duur van aanwezigheid van het virus in sperma, onderzochten wij dit in teruggekeerde symptomatische reizigers met een bevestigde ZIKV infectie (**Hoofdstuk 4**). Wij detecteerden ZIKV RNA middels RT-PCR in het sperma van negen van 15 deelnemers aan het onderzoek (60%). De mediane duur van ZIKV RNA detectie in sperma was 83 dagen (95%-betrouwbaarheidsinterval [57–108]) en de maximale duur van virus uitscheiding in semen in ons cohort was 144 dagen, na aanvang van de symptomen van ZIKV infectie. Wij verkregen slechts eenmaal een succesvolle isolatie van ZIKV uit semen, maar kunnen het potentieel voor seksuele overdracht niet uitsluiten zo lang als viraal RNA in semen gedetecteerd kan worden. Daarnaast vonden wij in de stalen van 11 deelnemers van wie het semen microscopisch werd onderzocht, leukocyten (n=11), erythrocyten (n=10) en oligospermie (n=6 van 10 (een van de deelnemers had een obstructieve azoöspermie na vasectomie)). Deze afwijkingen werden ook aangetroffen in stalen waarin geen ZIKV RNA werd aangetoond, en kunnen duiden op weefselschade aan de mannelijke voortplantingsorganen.

Bij de start van de ZIKV uitbraak in Latijns Amerika, waren slechts enkele diagnostische testen gevalideerd voor het aantonen van ZIKV infecties. Op het ITG werden symptomatische reizigers getest middels een ZIKV-specifieke RT-PCR op serum wanneer de stalen verkregen werden binnen 7 dagen na aanvang van de symptomen, en op urinestalen verkregen binnen 14 dagen na aanvang van de symptomen , en middels een anti-ZIKV Immuunglobuline (Ig)M en IgG Enzyme

Linked Immunosorbent Assay (ELISA). Alle positieve of twijfelachtige ELISA resultaten werden slechts als diagnostisch beschouwd voor ZIKV infectie na confirmatie in een Virus Neutralisatie Test (VNT), die werd uitgevoerd op de Eenheid Virologie van het Instituut. Asymptomatische reizigers werden alleen getest met ELISA op serum, bij voorkeur verkregen vanaf 20 dagen na terugkeer (d.w.z. de laatste blootstelling aan ZIKV in verband met de reis). In **Hoofdstuk 5** voerden wij een cross-sectionele cohort analyse uit om de hieroven geschetste benadering van ZIKV diagnostiek in niet-zwangere reizigers te evalueren. In een tijdsbestek van 12 maanden zagen wij 462 teruggekeerde reizigers op consultatie voor ZIKV diagnostiek. De diagnose ZIKV infectie werd bevestigd in 49, en kwam frequent voor onder symptomatische patiënten (46/227, 20.3%), maar niet bij asymptomatische personen (3/235, 1.3%). De asymptomatische reizigers hadden vergelijkbare karakteristieken met de symptomatische, maar zij hadden vaker zwangerschapswens (75.8% vs. 24.2%). Huiduitslag (positive likelihood ratio (LRP) 5.6) en conjunctivitis (LRP 10.8) voorspelden een diagnose van ZIKV infectie. De post-test probabiliteit van een negatief ELISA resultaat op serum verkregen op 20 tot 25 dagen na blootstelling was minder dan 0.1% in asymptomatische reizigers. Wij beschouwen negatieve ELISA resultaten op serum verkregen in dit tijdsbestek op 20 tot 25 dagen na blootstelling als een veilige strategie om ZIKV infectie uit te sluiten. Het advies om op 20 tot 25 dagen na blootstelling te testen voor de aanwezigheid van ZIKV-specifieke antilichamen kan bijzonder waardevol zijn voor het beleid bij terugkerende reizigers met een zwangerschapswens.

Scientific acknowledgements, conflict of interest statements and personal contribution

In this section I provide scientific acknowledgements consistent with the KU Leuven guidelines on fair and honest scientific conduct. In addition to details of ethics approvals, I have included co-authorship contributions that were defined according the recommendations of the International Committee of Medical Journal Editors (ICMJE) (http://www.icmje.org/recommendations/browse/roles-and-responsibilities/defining-the-role-of-authors-and-contributors.html).
For each chapter, author contributions and conflict of interest statements are listed. I also present a data availability statement for the studies in this thesis. Finally, I present a table of my personal contributions to the research.

Table 1. Overview of chapters in this PhD thesis

Chapt.#	Title	Title of manuscript
1.	Chikungunya virus infection and chronic joint pains	Chikungunya virus infection in Aruba: diagnosis, clinical features and predictors of post-chikungunya chronic polyarthralgia
2A.	A rapid test for chikungunya virus	Diagnostic accuracy of a rapid E1-antigen test for chikungunya virus infection in a reference setting
2B.		Variation at position 350 in the Chikungunya virus 6K-E1 protein determines the sensitivity of detection in a rapid E1-antigen test
3.	Traveling to 'Zika land'	Incidence of Zika virus infection in a prospective cohort of Belgian travelers to the Americas in 2016
4.	The potential for sexual transmission of Zika virus	Zika virus in semen: a prospective cohort study of symptomatic travellers returning to Belgium
5.	Zika virus infection in returning travelers	A cross-sectional analysis of Zika virus infection in symptomatic and asymptomatic non-pregnant travellers: experience of a European reference center during the outbreak in the Americas

Ethical approval

Ethical approvals for the studies in this thesis were obtained from the Institutional Review Board at the Institute of Tropical Medicine (ITM) and/or the Ethics Committee of the University Hospital Antwerp (UZA), Belgium, and (for the studies on chikungunya virus) the Board of the Horacio Oduber Hospital (HOH) in Aruba. The Institutional Review Board of ITM approved of an institutional policy of presumed consent for the use of de-identified and encoded patient data for research by consulting ITM physicians. Patients' identities were encoded by a unique study number and will not be released to anybody outside the medical and research team, except (and under confidentiality agreement) when necessary for the independent monitoring and auditing and in case of inspection by competent authorities. The prospective cohort studies were registered in a trial database (ClinicalTrials.gov).

Table 1. Overview of Ethics approvals and registration numbers

Chapter #	Ethical Committee	Date of Approval	Registration number	Registration ClinicalTrials.gov
1	ITM	2/6/2015	1013/15	
	UZA	13/07/2015	B300201525173	
	HOH			
2 (A&B)	ITM	2/6/2015	1013/15	
	UZA	13/07/2015	B300201525173	
	HOH			
3	ITM	25/11/2015	1045/15	
	UZA	25/01/2016	B300201627244	
				NCT02900066
4	ITM	11/03/2016	1081/16	
	UZA	21/03/2016	B300201628191	
				NCT02733796
5	ITM	Presumed consent		

ITM: Institutional Review Boards at the Institute of Tropical Medicine (ITM), Antwerp, Belgium
UZA: Ethics Committee of the University Hospital Antwerp
HOH: Board of the Horacio Oduber Hospital in Aruba

Author contributions per chapter

1. Chikungunya virus infection in Aruba: diagnosis, clinical features and predictors of post-chikungunya chronic polyarthralgia

Study conception:	Ralph Huits, Jan Jacobs, Emmanuel Bottieau, Lieselotte Cnops.
Methodology:	Ralph Huits.
Formal analysis:	Ralph Huits, Achilleas Tsoumanis, Marjan Van Esbroeck, Emmanuel Bottieau, Lieselotte Cnops.
Investigation:	Ralph Huits, Jaclyn De Kort, Riemsdijk Van Den Berg, Luis Chong, Kaat Eggermont, Koen Bartholomeeusen, Kevin K. Ariën, Marjan Van Esbroeck, Lieselotte Cnops.
Data curation:	Ralph Huits, Jaclyn De Kort, Riemsdijk Van Den Berg, Luis Chong, Kaat Eggermont, Lieselotte Cnops.
Resources:	Kevin K. Ariën, Emmanuel Bottieau, Lieselotte Cnops.
Writing initial draft:	Ralph Huits.
Visualization/ data presentation:	Ralph Huits.
Writing review & editing:	Ralph Huits, Jaclyn De Kort, Jan Jacobs, Marjan Van Esbroeck, Emmanuel Bottieau, Lieselotte Cnops
Supervision:	Jan Jacobs, Marjan Van Esbroeck, Emmanuel Bottieau, Lieselotte Cnops.
Project administration:	Ralph Huits.
Funding acquisition:	Marjan Van Esbroeck, Emmanuel Bottieau.

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Conflict of interest statement

All authors declare no conflict of interest.

2 A Diagnostic accuracy of a rapid E1-antigen test for chikungunya virus infection in a reference setting

Study conception:	Ralph Huits, Tamaki Okabayashi
Methodology:	Ralph Huits, Tamaki Okabayashi, Lieselotte Cnops, Barbara Barbé, Emi Nakayama, Tatsuo Shioda, Jan Jacobs
Formal analysis:	Ralph Huits, Tamaki Okabayashi, Lieselotte Cnops, Barbara Barbé, Emi Nakayama, Tatsuo Shioda
Investigation:	Ralph Huits, Barbara Barbé, Riemsdijk Van Den Berg, Koen Bartholomeeusen, Marjan Van Esbroeck, Lieselotte Cnops.
Data curation:	Ralph Huits, Barbara Barbé, Emi Nakayama, Tatsuo Shioda
Resources:	Kevin K. Ariën, Emmanuel Bottieau, Marjan Van Esbroeck.
Writing initial draft:	Ralph Huits.
Visualization/ data presentation:	Ralph Huits.
Writing review & editing:	Ralph Huits, Emmanuel Bottieau, Lieselotte Cnops, Koen Bartholomeeusen, Kevin K. Ariën, Marjan Van Esbroeck, Jan Jacobs,
Supervision:	Jan Jacobs, Marjan Van Esbroeck, Emmanuel Bottieau, Lieselotte Cnops, Tatsuo Shioda
Project administration:	Ralph Huits.
Funding acquisition:	Marjan Van Esbroeck, Emmanuel Bottieau, Tatsuo Shioda.

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ARKRAY Inc. (Kyoto, Japan) provided the E1-antigen test kits evaluated in this research. The company had no role in study design, data collection, and interpretation, or the decision to submit the work for publication.

Conflict of interest statement

All authors declare no conflict of interest.

2B. Variation at position 350 in the Chikungunya virus 6K-E1 protein determines the sensitivity of detection in a rapid E1-antigen test

Study conception:	Aekkachai Tuekprakhon, Emi E. Nakayama, Tatsuo Shioda, Pornsawan Leaungwutiwong
Methodology:	Aekkachai Tuekprakhon, Emi E. Nakayama, Koen Bartholomeeusen, Orapim Puiprom, Tadahiro Sasaki, Ralph Huits, Tatsuo Shioda, Pornsawan Leaungwutiwong
Formal analysis:	Aekkachai Tuekprakhon, Emi E. Nakayama, Koen Bartholomeeusen, Orapim Puiprom, Ralph Huits, Natthanej Luplertlop, Nathamon Kosoltanapiwat, Pannamas Maneekan, Kevin K. Ariën, Tatsuo Shioda, Pornsawan Leaungwutiwong
Investigation:	Aekkachai Tuekprakhon, Emi E. Nakayama, Koen Bartholomeeusen, Orapim Puiprom, Ralph Huits, Natthanej Luplertlop, Nathamon Kosoltanapiwat, Pannamas Maneekan, Kevin K. Ariën, Tatsuo Shioda, Pornsawan Leaungwutiwong
Data curation:	Aekkachai Tuekprakhon, Emi E. Nakayama, Koen Bartholomeeusen, Orapim Puiprom, Ralph Huits, Natthanej Luplertlop, Nathamon Kosoltanapiwat, Pannamas Maneekan, Kevin K. Ariën, Tatsuo Shioda, Pornsawan Leaungwutiwong
Resources:	Emi E. Nakayama, Koen Bartholomeeusen, Natthanej Luplertlop, Kevin K. Ariën, Tatsuo Shioda, Pornsawan Leaungwutiwong
Writing initial draft:	Aekkachai Tuekprakhon, Emi E. Nakayama
Visualization/ data presentation:	Aekkachai Tuekprakhon, Emi E. Nakayama, Koen Bartholomeeusen, Orapim Puiprom
Writing review & editing:	Aekkachai Tuekprakhon, Emi E. Nakayama, Koen Bartholomeeusen, Orapim Puiprom, Tadahiro Sasaki, Ralph Huits, Natthanej Luplertlop, Nathamon Kosoltanapiwat, Pannamas Maneekan, Kevin K. Ariën, Tatsuo Shioda, Pornsawan Leaungwutiwong
Supervision:	Emi E. Nakayama, Natthanej Luplertlop, Nathamon Kosoltanapiwat, Pannamas Maneekan, Tatsuo Shioda, Pornsawan Leaungwutiwong
Project administration:	Tatsuo Shioda, Pornsawan Leaungwutiwong
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Conflict of interest statement

All authors declare no conflict of interest.

3. Incidence of Zika virus infection in a prospective cohort of Belgian travellers to the Americas in 2016

Study conception:	Ralph Huits , Jan Jacobs, Emmanuel Bottieau
Methodology:	Ralph Huits , Dorien Van Den Bossche, Lieselotte Cnops, Emmanuel Bottieau
Formal analysis:	Ralph Huits, Achilleas Tsoumanis
Investigation:	Ralph Huits , Kaat Eggermont, Erica Lotgering, Marjan Van Esbroeck, Lieselotte Cnops
Data curation:	Ralph Huits
Resources:	Ralph Huits, Marjan Van Esbroeck, Emmanuel Bottieau
Writing initial draft:	Ralph Huits
Visualization/ data presentation:	Ralph Huits
Writing review & editing:	Ralph Huits , Dorien Van Den Bossche, Kaat Eggermont, Erica Lotgering, Anne-marie Feyens, Idzi Potters, Jan Jacobs, Marjan Van Esbroeck, Lieselotte Cnops, Emmanuel Bottieau
Supervision:	Jan Jacobs, Marjan Van Esbroeck, Lieselotte Cnops, Emmanuel Bottieau
Project administration:	Ralph Huits , Erica Lotgering, Lieselotte Cnops
Funding acquisition:	Ralph Huits, Marjan Van Esbroeck, Emmanuel Bottieau

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Conflict of interest statement

All authors declare no conflict of interest.

4. Zika virus in semen: a prospective cohort study of symptomatic travellers returning to Belgium

Study conception:	Ralph Huits, Lieselotte Cnops
Methodology:	Ralph Huits, Lieselotte Cnops
Formal analysis:	Ralph Huits, Birgit De Smet, Kevin K. Ariën, Marjan Van Esbroeck, Lieselotte Cnops
Investigation:	Ralph Huits, Birgit De Smet, Kevin K. Ariën, Marjan Van Esbroeck, Lieselotte Cnops
Data curation:	Ralph Huits, Lieselotte Cnops
Resources:	Marjan Van Esbroeck, Emmanuel Bottieau
Writing initial draft:	Ralph Huits
Visualization/ data presentation:	Ralph Huits, Achilleas Tsoumanis
Writing review & editing:	Ralph Huits, Birgit De Smet, Kevin K. Ariën, Marjan Van Esbroeck, Emmanuel Bottieau, Lieselotte Cnops
Supervision:	Marjan Van Esbroeck, Emmanuel Bottieau, Lieselotte Cnops
Project administration:	Ralph Huits, Lieselotte Cnops
Funding acquisition:	Ralph Huits, Marjan Van Esbroeck, Emmanuel Bottieau

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Conflict of interest statement

All authors declare no conflict of interest.

5. A cross-sectional analysis of Zika virus infection in symptomatic and asymptomatic non-pregnant travellers: experience of a European reference center during the outbreak in the Americas

Study conception:	Ralph Huits, Emmanuel Bottieau
Methodology:	Ralph Huits
Formal analysis:	Ralph Huits, Achilleas Tsoumanis
Investigation:	Ralph Huits, Ula Maniewski, Dorien Van Den Bossche, Lieselotte Cnops, Marjan Van Esbroeck, Emmanuel Bottieau
Data curation:	Ralph Huits, Erica Lotgering, Lieselotte Cnops
Resources:	Marjan Van Esbroeck, Emmanuel Bottieau
Writing initial draft:	Ralph Huits
Visualization/ data presentation:	Ralph Huits
Writing review & editing:	Ralph Huits, Ula Maniewski, Dorien Van Den Bossche, Erica Lotgering, Achilleas Tsoumanis, Lieselotte Cnops, Jan Jacobs, Marjan Van Esbroeck, Emmanuel Bottieau
Supervision:	Jan Jacobs, Emmanuel Bottieau
Project administration:	Ralph Huits, Ula Maniewski, Dorien Van Den Bossche, Marjan Van Esbroeck
Funding acquisition:	Ralph Huits, Emmanuel Bottieau

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The funding organizations had no role in the study design, in the collection, analysis and interpretation of data, in the writing of the manuscript, and in the decision to submit the manuscript for publication.

Conflict of interest statement

All authors declare no conflict of interest.

Data Availability Statement for all studies

The data supporting the findings of this study/publication are retained at the Institute of Tropical Medicine, Antwerp and will not be made openly accessible due to ethical and privacy concerns. Data can however be made available after approval of a motivated and written request to the Institute of Tropical Medicine at ITMresearchdataaccess@itg.be.

Role of PhD candidate

My role in the different components of my PhD thesis is listed in the table below. I used the 'Taxonomy categories' and 'Descriptions of role' as proposed by Liz Allen et al. Nature. 2014; 508(7496):312-3.

Taxonomy category	Description of role	1	2A	2B	3	4	5
Study conception	Ideas; formulation of research question; statement of hypothesis.	+	+	+	+	+	+
Methodology	Development or design of methodology; creation of models.	+	s	+	s	+	+
Computation	Programming, software development; designing computer programs; implementation of the computer code and supporting algorithms.	+	+	-—	+	s	+
Formal analysis	Application of statistical, mathematical or other formal techniques to analyze study data.	s	+	+	s	s	+
Investigation: performed the experiments	Conducting the research and investigation process, specifically performing the experiments.	-—	s	s	-—	-—	-—
Investigation: data/evidence collection	Conducting the research and investigation process, specifically data/evidence collection.	+	+	+	+	+	+
Resources	Provision of study materials, reagents, materials, patients, laboratory samples, animals, instrumentation or other analysis tools.	s	s	s	+	s	S
Data curation	Management activities to annotate (produce metadata) and maintain research data for initial use and later re-use.	+	+	-—	+	+	+
Writing/manuscript preparation: initial draft	Preparation, creation and/or presentation of the published work, specifically writing the initial draft.	+	+	-—	+	+	+
Writing/manuscript: critical review, commentary or revision	Preparation, creation and/or presentation of the published work, specifically critical review, commentary or revision.	s	s	s	s	s	S
Writing/manuscript: visualization/data presentation	Preparation, creation and/or presentation of the published work, specifically visualization/data presentation.	+	+	S	+	+	+
Supervision	Responsibility for supervising research; project orchestration; principal investigator or other lead stakeholder.	+	+	-—	s	+	+
Project administration	Coordination or management of research activities leading to this publication.	+	+	-—	+	+	+
Funding acquisition	Acquisition of the financial support for the project leading	s	s	-—	-—	-—	NA

\+ *denotes role of the PhD candidate* S *denotes shared role of the PhD candidate*
-— *denotes role of co-researcher* NA *Not Applicable*

Curriculum vitae

Ralph Martin Henri Gerard Huits was born on February 23rd 1969 in Eindhoven, the Netherlands. He obtained his Gymnasium diploma from the Augustinianum secondary school in Eindhoven in 1987. He studied Medicine (1987-1994) at Maastricht University.

He trained as a Tropical Doctor in a program supervised by the Netherlands Society for Tropical Medicine and International Health. In 1997, he obtained a Diploma in Tropical Medicine and Hygiene (DTM&H) from the Royal Tropical Institute in Amsterdam, the Netherlands and a Post Graduate Diploma in Infectious Diseases from the London School of Hygiene and Tropical Medicine. From 1998 to 2001 he worked as a Medical Officer at Willis F. Pierce Memorial Hospital (Mount Selinda Mission Hospital) in Chipinge District, Zimbabwe.

From 2001, he trained in Clinical Microbiology and worked in an intensive care unit at the University Medical Center Groningen, the Netherlands. He specialized in Internal Medicine in Groningen under the supervision professor R.O.B. Gans, and at the University Medical Center St. Radboud in Nijmegen under the supervision of professor J.W.M. van der Meer and professor J. de Graaf. He completed his Infectious Diseases Fellowship at University Medical Center St. Radboud under the supervision of professor B.J. Kullberg, and registered as an Internist and Infectious Disease specialist in 2009. He obtained a certificate in diagnostic gastroscopy and colonoscopy at the Catharina Hospital in Eindhoven.

From 2010 to 2012, he worked at the Horacio E. Oduber Hospital in Aruba (Dutch Caribbean), where he coordinated an antimicrobial stewardship program, HIV and hepatitis care. After 2010's earthquake in Haiti, he participated in an emergency relief mission to Port-au-Prince, coordinated by the Aruba Red Cross.

Throughout his professional career, he has been dedicated to teaching nursing staff, undergraduates, graduates and peers. In 2012 he obtained the basic teaching qualification (Basis Kwalificatie Onderwijs) from the VU University Medical Center in Amsterdam.

In 2014 he took up his current position as an internist at the Unit of Tropical Medicine in the Department of Clinical Sciences of the Institute of Tropical Medicine (ITM) in Antwerp, Belgium, headed by professor E. Bottieau, and as a consultant in Tropical Medicine to the University Hospital of Antwerp. He had developed a research interest in emerging arbovirus infections, when studying predictors of severe dengue virus infections in Aruba. He started his PhD under the supervision of professor J.A. Jacobs (ITM/ KU Leuven) and professor E. Bottieau (ITM). The title of his PhD thesis is 'Challenges in diagnosis and management of Chikungunya and Zika virus infections'.

Dr. Huits served as an external advisor to the WHO 'Sexual Transmission of Zika virus Expert Meeting'. At ITM, he was a member of the working party on authorship ethics of ITM's Scientific Integrity Commission. As a member of the ZikaPLAN consortium, his proposal for studying the persistence of Zika virus in semen was awarded a grant from the European Union's Horizon 2020 research and innovation programme.

Ralph Huits is married to Suzanne Lenaerts.
They have two children, Milan and Busi.

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Publications in the context of this thesis

Van den Bossche D, Michiels J, Cnops L, Foque N, Meersman K, Huits R, Ariën KK, Van Esbroeck M. Challenges in diagnosing Zika – experiences from a reference laboratory in a non-endemic setting. (accepted for publication in European Journal of Clinical Microbiology & Infectious Diseases)

Tuekprakhon A, Puiprom O, Sasaki T, Meno MK, Nakayama EE, Phadungsombat J, Bartholomeeusen K, Huits R, Ariën KK, Luplertlop N, Shioda T, Leaungwutiwong P. Broad-spectrum monoclonal antibodies against chikungunya virus structural proteins: promising candidates for antibody-based rapid diagnostic test development (accepted for publication in PLoS ONE)

Huits R, Van den Bossche D, Eggermont K, Lotgering E, Feyens A, Potters I, Jacobs J, Van Esbroeck M, Cnops L, Bottieau E. Incidence and characteristics of Zika virus infection in adult Belgian travellers to the Americas in 2016: a case-cohort study. Int J Infect Dis. 2018 Oct 24. pii: S1201-9712(18)34556-9.

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Huits R, Van den Bossche D, Bottieau E, Van Esbroeck M. Re: Lack of Zika virus antibody response in confirmed patients in non-endemic countries. J Clin Virol. 2018 Jul 17;106:33.

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Huits RMHG, De Smet B, Ariën KK, Van Esbroeck M, de Jong BC, Bottieau E, Cnops L. Kinetics of Zika virus persistence in semen. [Submitted]. Bull World Health Organ. E-pub: 06 Jul 2016.

PhD-related Presentations

November 3 2015	"Self-testing by travellers: why malaria rapid diagnostic tests differ from pregnancy tests" (CME at ITM, Antwerp)
February 23 2016	The Guardian (global-development-professionals-network) Expert panel on Zika virus: https://www.theguardian.com/global-development-professionals-network/2016/feb/23/the-fight-against-Zika-we-need-a-proactive-global-health-agenda-expert-panel

March 11 2016	ITM special seminar Zika “Natural history, Clinical signs and Symptoms” April 9-12 2016 26th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID 2016). “Epidemiology of Guillain-Barré syndrome on Aruba” (Poster presentation EV0999)
May 10-13 2016	Conference on Molecular Epidemiology and Evolutionary Genetics of Infectious Diseases, Institute of Tropical Medicine, Antwerp, Belgium “Zikavirus 2016: Public Enemy No. 1?” (Invited plenary session talk)- 13th International
March 21-22 2017	Sexual Transmission of Zika virus Expert Meeting, Geneva (WHO external advisor)
March 23 2017	Congres Belgische Vereniging voor Kindergeneeskunde “Clinically important and imported arbovirus infections”. (Invited Plenary lecture)
May 14-18 2017 15th	Conference of International Society of Travel Medicine, Barcelona, Spain “Post-Exposure Screening For Zika Virus Antibodies In Asymptomatic Travellers Who Want To Conceive” (Oral presentation, 16/05/2017) “Clinical Presentation Of Zika Virus Infection In Belgian Travellers Returning From The Americas” Poster presentation (PO14.11) Poster Tour Leader

October 16-20 2017	10th European Congress on Tropical Medicine and International Health (ECTMIH), Antwerp, Belgium "Incidence of Travel Associated Zika Virus Infection in 2016: Preliminary Results of a Prospective Cohort Study in Belgian Travellers To The Americas" (Oral presentation) "Clinical Features of Chikungunya Infection and Chronic Polyarthralgia after the 2014-2015 Outbreak on Aruba" (Oral presentation)
November 7 2017	66th American Society of Tropical Medicine and Hygiene annual meeting, Baltimore, USA "Persistence Of Zika Virus In Semen Of Men Living In An Endemic Area" Poster presentation (#825)
February 5-6 2018	Coalition for Epidemic Preparedness (CEPI) workshop on 'Chikungunya Vaccines-Challenges, Opportunities and Possibilities', New Delhi, India. "The importance of good diagnostics" Oral presentation (invited)
June 4-6 2018	International Symposium on Zika Virus Research, Marseille, France Sexual transmission of Zika virus: the current state of affairs (Key-note lecture, invited)

Other publications

Ralph Huits, Gonda De Ganck, Jan Clerinx, Philippe Büscher, Emmanuel Bottieau. A veterinarian with fever, rash and chancre after holidays in Uganda. J Travel Med. 2018 Oct 17. (Epub ahead of print)

Theunissen C, Cnops L, Van Esbroeck M, Huits R, Bottieau E. Acute-phase diagnosis of murine and scrub typhus in Belgian travelers by polymerase chain reaction: a case report. BMC Infect Dis. 2017 Apr 13;17(1):273.

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Posters and presentations

Serum Ferritin as a Predictor of Outcome in Dengue Virus Infection (Poster) Infectious Diseases Society of America meeting in 2011, Boston

Van de Weg C, Huits R, Brouns M, van den Berg R, Martina B, Osterhaus A, E Van Gorp. Elevated ferritin levels in dengue virus infected patients from Aruba. International Journal of Infectious Diseases vol. June 16, 2012. p. E116. -Presentation at 15th International Congress on Infectious Diseases, Bangkok, Thailand

Suryapranata FST, Ang CW, Chong LL, Murk J, Falconi J, Huits RM
The epidemiology of Guillain-Barré syndrome on Aruba- ePoster at ECCMID 2016, Amsterdam

Other scientific activities

Ralph Huits is a reviewer for multiple scientific journals, including:
The American Journal of Tropical Medicine and Hygiene, Clinical Infectious Diseases, Clinical Microbiology and Infection, Plos One, Emerging Infectious Diseases, The Journal of Infectious Diseases, International Journal of Infectious Diseases, Nederlands Tijdschrift Voor Geneeskunde, Reproductive BioMedicine Online, Clinical Rheumatology, BMJ Open, and BMJ Case Reports.

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