Come Fly With Me:

Integration of travel medicine and arbovirus surveillance

Natalie Bea Cleton

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Come Fly With Me

Integration of travel medicine and arbovirus surveillance

Vlieg met me mee

Integreren van reizigersgeneeskunde met arbovirus surveillance

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MUSKIETE JAG
A.D. Keet

Jou vabond, wag, ek sal jou kry,
Van jou sal net 'n bloedkol bly
    Hier teen my kamermure.
Deur jou vervloekte gonsery,
Deur jou gebyt en plagery,
    Kon ek nie slaap vir ure.

Mag ek my voorstel, eer ons skei,
Eer jy die doodslag van my kry –
    My naam is van der Merwe.
Muskiet, wees maar nie treurig nie,
Wees ook nie so kieskeurig nie.
    Jy moet tog ééndag sterwe.

Verwekker van malaria,
Sing maar jou laaste aria –
    Nog een minuut vir grasie.
Al soebat jy nou nog so lang,
Al sê jy ook: ek is nie bang,
    Nooit sien jy weer jou nasie…

Hoe sedig sit hy, o die kreng!
Sy kinders kan maar kranse bring,
    Nòu gaan die vabond sterwe…..
Pardoef! Dis mis! daar gaan hy weer!
Maar dóód sal hy, sowaar ek sweer –
    My naam is van der Merwe.

1 |

1

General Introduction

General introduction

Arboviruses are a group of viruses that use arthropod vectors as their main transmission route and are therefore defined as ARthropod-BOrne viruses. Known vectors include mosquitoes, ticks, midges, and sandflies. They transmit viruses belonging to various families that are taxonomically unrelated, but grouped by their shared biomechanical characteristic. Of the over 545 suspected arbovirus species, more than 150 cause disease in humans, and most of these are zoonotic.[1-3]

Most pathogenic human arboviruses are sustained in a transmission cycle in which the vectors are arthropods, mainly mosquitoes, and the animal reservoirs are mainly domestic poultry, wild birds, and rodents that serve as the amplifying hosts (figure 1). An amplifying host develops a high level of viremia for sufficient time to infect the vectors that feed on it. Humans are often dead-end hosts, as they seldom develop the high viremias needed to infect arthropods. A few viruses like yellow fever virus (YFV), chikungunya virus (CHIKV), and dengue virus (DENV) have expanded their host range to include humans as an amplifying host, thereby losing the need for an animal reservoir to sustain virus circulation.[4-7]

Several factors determine the capacity of blood-feeding (*hematophagous*) arthropods to serve as vectors in a transmission cycle. First, certain animal hosts must be available to the type of arthropod that feeds on them.[8] Second, the arthropod must be competent to become infected by feeding on a viremic host. Third, the virus must be able to survive digestion by the arthropod; it must then replicate and be disseminated from the salivary glands when the arthropod feeds on a susceptible and uninfected host.[8, 9] Of all arthropods, mosquitoes form the largest and most important group of vectors for arboviruses.[8]

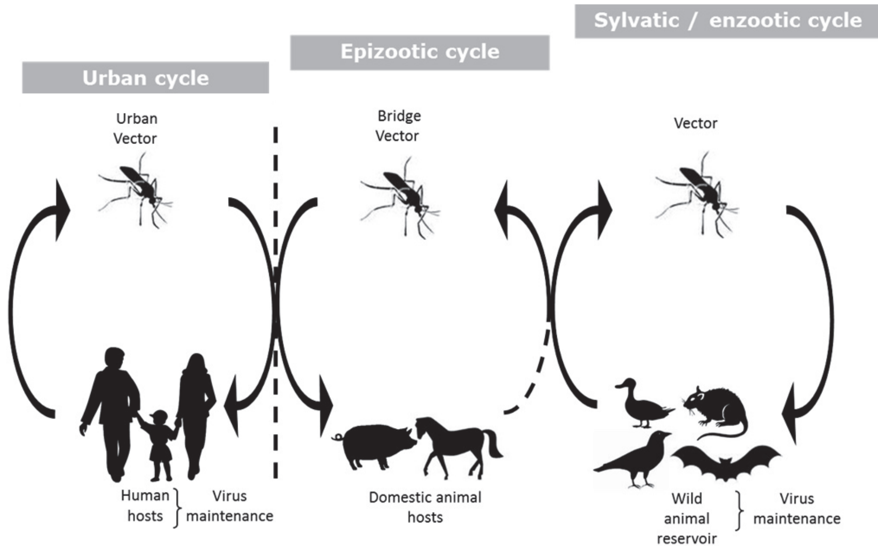
In the sylvatic cycle arboviruses survive by transmission between specific wild animals and specific arthropods. Humans are accidental hosts that become infected by entering the sylvatic cycle through agriculture, urbanisation, or outdoor leisure activities.[10-12] These human incursions change the natural environment of the virus and potentially lead to numerous secondary vectors that can serve as bridge vectors, by feeding on infected birds and wild mammals and in turn infecting domestic animals and humans. This process can turn an enzootic cycle into an epizootic or epidemic cycle. Viruses that use humans as their main reservoir, like CHIKV and DEN, are thought to have spilled over from a sylvatic cycle. They are now sustained mainly in an urban or suburban population in which mosquitoes transmit viruses directly from one person to the next.[10, 11, 13, 14]

The emergence of arboviruses in Europe

Recent decades have seen increasing arboviral outbreaks caused by importation of new virus species to Europe or the spread of established species to new parts of Europe.[15-18] In 2001, Usutu virus, probably introduced by bird migration from African continent was diagnosed as the cause of avian deaths across Austria.[20] The 2011 emergence of a new disease in cattle named Schmallenberg virus caught the Dutch/European animal production industry by surprise, causing major stock and monetary losses.[19] Arboviruses already present in Europe that are expanding their territory include West Nile virus (WNV), which

since 1996 has caused outbreaks of encephalitis in horses and humans in France, Greece, Italy, Romania and Hungary.[21-25]

Figure 1: General cycle arboviruses



Many factors interact to shape the likelihood of disease outbreaks. Among others, these driving factors include vectors, human and animal population densities, climate variation, and habitat fragmentation.[11, 26] Understandably, their disparity makes disease surveillance and outbreak predictions challenging. The emergence of new diseases and changes of circulation of current diseases are intertwined with these factors through introduction of vectors, animals, or humans into new areas and through mutations that result in adaptation to a new vector and/or increased fitness of virus or host. Additionally, improvements in detection capability could reveal diseases that appear emergent but were actually present without being detected.

Globalisation plays an important role in disease emergence through rapid introduction of viruses into naïve populations. In addition to the Schmallenberg and Usutu viruses, recent examples are the introduction and continued transmission of DENV and CHIKV into Europe and the current CHIKV and Zika virus outbreaks in the Americas.[27-32]

Over the last century, international travel and trade have expanded due to easier air traffic, the speed and declining cost of human and/or freight transport, the concentration and movement of cheap labour, and development of new markets and products around the world. Billions of animals are legally transported worldwide for food, food production, as pets, for science, and conservation.[27] The number increases dramatically when illegal trade is included. The importation of the 'tiger mosquito' *Aedes Albopictus* through Lucky Bamboo plants from China has substantiated the risk of introducing exotic vectors through trade in Europe and the Netherlands.[33] This mosquito is a competent vector for the CHIKV and DENV circulating in China, and can serve as vector for a range of arboviruses.

Not only animals and products but also humans are increasingly travelling around the world; for work, immigration, tourism, or visiting family and friends. In 2014, Schiphol airport in the Netherlands served 45.2 million passengers, of whom 26.4 million had Schiphol as their final destination or starting point. The increasing amount and speed of travel raises the risk of travellers becoming infected with exotic diseases, and viremic passengers have increasingly brought CHIKV into Europe. [34-37] For several years, the lack of an appropriate vector kept the virus from gaining a foothold, but in 2007, a passenger from India brought to Italy a new CHIKV variant that could replicate much better in *Aedes albopictus* than other CHIKV viruses. This mosquito is not native to Europe but has expanded its European population, over the past decade, since being imported in old tires containing its eggs.[38, 39] These two factor, the importation of a new CHIKV variant and the previous importation of *A. albopictus*, made the CHIKV outbreak in Italy in 2007 and France in 2010 possible.[11, 40]

In 2011-2013, 80 percent of the Dutch population booked one or more vacations annually, spending about 50 percent abroad, or about 18 million vacations each year.[41] Although Europe remains the main destination, more and more Dutch travellers seek alternative destinations like the Far East, the Caribbean, Asia and Africa.[41] The expanding worldwide outbreaks of DENV, especially after 2000, and of CHIKV in Asia after 2004, have brought more attention to the possible presence of these infectious diseases in travellers, spurring development of more preparedness schemes focussing on travellers.[28, 29, 34-36, 42, 43]

Recent risk-mapping models that take habitat, climate, host animals, pathogens, and socio-economic factors into account indicate that parts of Europe, including the Netherlands, are at potential risk for emerging infectious diseases, specifically zoonotic diseases.[1, 44] As yet, however, only very limited surveillance is performed in the Netherlands, and no arboviruses have been found to be circulating in local wildlife or the general human population.[3, 16, 45]

Surveillance

Multiple surveillance programs have been initiated, some of which use travellers to assess the risk of imported pathogens to individual and public health.[46-49] The two main programs that provide annual reports of disease in travellers are the Geosentinel and EuroTravNet networks.[46] However, only a few clinics participate; the data must be submitted manually, and information is not reported in real time but collected for focussed reports spanning several years.[46, 47] Although these networks show the value of surveillance in travellers, much diagnostic data remains unused and dependent on retrospective reporting. Accordingly, we explored whether routine laboratory arbovirus submission data and results could be used in additional and possibly real-time monitoring of illness trends, with international travellers contributing to surveillance of individual health and both national and international public health.[49]

Goal, outline of thesis, and research questions

Given the diversity and unpredictability of emerging diseases and the difficulty of identifying which will be a future threat, innovative preparedness strategies to focus on multiple pathogens, species, and symptoms are needed. Current arbovirus surveillance in Europe is patchy and often focussed on a single disease. Also, it is typically separate from the general health structures, as the information systems and data used in the medical field are not

optimal for use in surveillance.[50] Going forward, however, the economic burden of surveillance of so many diseases, as well as preparing for emerging diseases, requires moving away from the paradigm of focussed single-disease surveillance based on optimal data. For sustainable emerging disease preparedness, pre-existing information sources may provide effective surveillance opportunities.[49]

In this thesis, we focus on integrating travel medicine with public health surveillance, using travellers as sentinels for arbovirus activity within the global health community. We investigate possibilities to utilise our current hospital information systems, using the anonymised data collected for individual patient care of travellers, to conduct real-time national and international surveillance. However, as pathogen exposure and outbreaks often occur in animals before humans, certain animals are suitable as sentinels and can play an essential part of arbovirus surveillance.[51-53] We therefore explored possibilities in veterinary medicine, exploring the One Health perspective in arbovirus surveillance.

The overall goal of this thesis is to study how existing medical information and medical health structures could be used for arbovirus surveillance in the Netherlands for the identification and quantification of arbovirus threats to public and veterinary health.

To achieve this goal, laboratory-based tools and epidemiological models are integrated and used for risk and exposure profiling to obtain crucial information on arboviral exposure, circulation, and transmission. The general steps within the overall project can be divided into three main research questions addressed in chapters two through four.

In **chapter two** we investigate whether information from routine diagnostic databases can be used [as a resource] in surveillance of the arboviral disease burden in travellers.

In **chapter three** we quantify which arboviruses pose a travel risk and how they can be categorised to provide systematic information for diagnostics and surveillance.

In **chapter four** we investigate how arbovirus diagnostics are currently performed in the Netherlands and to what extent travellers are at risk of under-diagnosis?

In **chapter five** we develop an arbovirus protein micro-array for multiplex serological diagnosis of arbovirus infections in humans (imported by travellers) and equines (local circulation).

In **chapter six** we perform a retrospective study in travellers to evaluate the added value of standardised algorithms and multiplex testing of arboviruses for public health surveillance and patient care.

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2

21

Using routine diagnostic data as a method of surveillance of arboviral infection in travellers: a comparative analysis with a focus on dengue

Cleton N., Reusken C., Murk JL., De jong M., Reimerink J., Van der Eijk A. and Koopmans M.

Abstract

Background: In a large part of the developing world, limited infectious disease surveillance is performed. In laboratory information management systems data on diagnostic requests is available and may be amenable to trend analyses. We explored this potential, using DENV diagnostic requests as a model.

Method: Test results and anonymised information provided by clinicians were received for 8,942 patients from diagnostic centres in the Netherlands from January 2000 to May 2011. The data were evaluated for completeness of a predefined minimal dataset and trends in DENV positive results by travel destination. Population travel data were obtained from a commercial registry, and dengue case notification data by country from WHO DengueNet.

Results: Vaccination history was rarely reported (0.4%); travel destination was completed for 42% of requests; trends in diagnostic requests and IgM positive tests for this subset correlated to the WHO DENV notifications for the three main travel destinations, with some discrepancies. Additionally, this approach may provide information on disease outbreaks with other pathogens causing diseases clinically similar to DENV. PCR data proved to be insufficient for trend monitoring by country.

Conclusion: This approach is not straightforward, but shows potential for use as a source of additional information for surveillance of disease.

Travel Medicine and Infectious Diseases, 2014 Mar-Apr;12(2):159-66

Introduction

New diseases continue to emerge across the world, due to a complex array of factors relating to demographics, increasing demand for animal protein, deforestation, and a steep increase in international travel and trade [1, 2]. Vector-borne diseases, with the exception of West Nile and Dengue virus, are considered neglected tropical diseases and no or little surveillance is performed in a large part of the developing world.

Systematic evaluation of health complaints through travel clinics has shown a high incidence of health complaints (8-10%) [3, 4]. As a consequence, international travellers can be seen as sentinels and sources of introduction for infectious diseases occurring worldwide [5-8]. Developing diagnostic tools and approaches to monitor health complaints in returning travellers could provide an interesting addition to traditional surveillance [8].

Geosentinal clinics form an international network that collects data on diseases in travellers. However, the number of clinics participating is limited and the reporting system depends on the extent to which clinicians actively upload information. A large amount of diagnostic data is therefore available in diagnostic laboratories but remains unused for surveillance purposes. This had led us to explore the use of routine laboratory submission data and results for additional monitoring of trends of illness through international travellers. Here, we set out to explore this potential by using dengue virus as an example.

Dengue virus (DENV) is considered one of the most important arboviruses globally, with 2.5 billion people at risk of infection according to the World Health Organization (WHO) [9]. It is a well-recognized disease in travellers to tropical and subtropical regions and therefore diagnostic tests are requested frequently [10-12]. Surveillance is done in many countries that are popular travel destinations, and DENV probable and confirmed cases are notified on voluntary basis to the WHO. This currently provides the best insight into the DENV situation per country and therefore offers a potential comparison and addition to data provided by diagnostic laboratories [13, 14]. Routine diagnostic information in travellers coupled to travel history and symptoms could potentially be used as an already in-place cost-effective additional information source for monitoring the demographics of disease and exposure trends where no surveillance in available [15].

The goal of our study is to investigate the usability of diagnostic information of returning travellers on travel history, symptoms and diagnostic results provided by routine diagnostic laboratories as complementary information usable in surveillance for disease in travellers, providing information on public health threats by possible introduction of viremic patients and trends in local disease activity. We use DENV as an example since WHO surveillance information is available for some countries as comparison and complementary data.

Methods

Diagnostic data: During a consensus meeting between the diagnostic laboratories and responsible researchers, a minimal dataset needed for data analysis was proposed and discussed based on the question what minimal information was essential for use in surveillance of disease in travellers and the countries they visit. The defined minimum dataset was age, sex, travel date and destination, description of clinical symptoms, vaccination history, diagnostic results and test(s) used.

Age and sex were considered to be of importance in order to identify risk groups and make results compatible between diagnostic centres. Information of *travel history and dates* were needed to correlate demographic distribution of infections in Dutch travellers to current known and unknown outbreaks. This information was also needed for interpreting results as new or old infections and possible cross-reactions with co-circulating cross-reactive arboviral infections [16-19]. Records of *clinical symptoms* were needed to evaluate the usefulness of the clinical data for syndromic surveillance [20]. Finally, *vaccination history* was considered essential for the data analysis since a number of flavivirus vaccinations (TBEV, JEV, YFV) are known to cross-react causing false positive IgG diagnostic results [16, 19].

Data containing the diagnostic results, interpretation, and the information provided by clinicians with the requests for DENV diagnostics were retrospectively extracted from the laboratory information management systems (LIMS) from the three main arboviral diagnostic labs in the Netherlands from 2000 to 2011. This represents the vast majority of all DENV diagnostic requests in the Netherlands.

The information was provided in excel format as raw data. As there is no standardized testing for DENV infections, each laboratory provided the interpretations of the results. Diagnosis was based on determination of IgM and IgG antibodies by rapid lateral flow Immunochromatographic Test (ICT) by Panbio (Brisbane, Australia) (one laboratory), immunofluorescence assay (IFA) by Progen (Heidelberg, Germany) and Scimedix (Denville, New Jersey, USA) (one laboratory) and Enzyme-linked immunoassays (ELISA) from Focus (Cypress, CA, USA) (two laboratories). Each laboratory provided a cut-off for defining if a diagnostic result was considered positive or negative. Laboratories using IFA and ELISA tests used a higher but comparable cut-off than recommended by the manufacturer to increase specificity. These cut-offs were defined by validation prior to implementation of the assays in the diagnostic routine. In compliance with the medical ethical guidelines, no personal identifiers were included.

Data analysis: A database was created in Microsoft Excel (edition 2007). The raw data was organized and imported into "R" (version 2.14.0) for further analysis. The data was evaluated for completeness of the minimal defined dataset and for trends in DENV positive results by travel destination. These trends were compared with those based on data

collected by WHO DengueNet 2001 to 2011 that are published by the WHO regions online (South-East Asia only published data until 2010).

Travel data: Data were obtained from a commercial research registry "ContinuVakantieOnderzoek" (CVO) that follows travel trends by interviewing, every three months, 10,000 out of a panel of 150,000 Dutch homes about their travel behaviour [21]. The research has been conducted since the 1980's and is used for monitoring Dutch travel behaviour in the tourist and travel industry. Basic information is requested on number and length of vacations within and outside the country (if any), location and lodging, activities, method of transport and booking. Information is analyzed in condensed form for reports and raw data is available in an online database HolidayTracker [21].

Estimation of the proportion of DENV infections in travellers, 2010: As travellers may be seen as a source of introduction of arboviral infections, we tried to use the available data to provide an estimate range for the possible proportion of travellers infected with Dengue during one year. For this, the lower limit estimate was made by

$E_{low} = (P*4)/N$

Where E_{low} is the lower estimate of DENV infections, P is the number of DENV IgM positive patients, 4 is the multiplication factor assuming 25% of all cases are symptomatic [22], and N is the number of travellers to DENV endemic countries.

The higher estimate was made by:

$E_{high} = ((N*0.1)*(P/T))/N*4$

Where 4, N and P are as indicated above, 0.1 is the estimated proportion of travellers returning with febrile illness [3, 4, 23, 24], and T is the number of samples tested.

Statistical analysis: Proportions were tested with Pearson's chi-squared test for equality of proportions. Trend lines were tested with F-test and R^2 and correlations were calculated using Spearman's rank correlation coefficient for describing independence between variables (r_s).

Results

Completeness of diagnostic data

In total, data were received on 10540 diagnostic samples from 8,942 patients from the three diagnostic centres in the Netherlands from January 2000 until May 2011. Each laboratory provided diagnostic result and interpretation per patient.

The completeness of data ranged from 0.5% for vaccination history to 98% and 99% for sex and birth date. Some basic clinical information was provided with 75% of requests, but only 42% of the requests mentioned information on travel history (Table 1). Only 0.2% of diagnostic requests contained all parameters.

Trends in diagnostic requests and results

Between 2000 and 2011 RT-PCR for detection of DENV viral RNA was performed on 275 samples (3%) of DENV diagnostic requests. More than 50% of the RT-PCR requests have occurred in the last three years and the percentage of positive samples has dropped from 30% to 13% (data not shown). RNA was more often detected in IgM positive (23%) than in IgM negative patients (9%).

The annual number of serological diagnostic requests for DENV has increased significantly over time (Figure 1). In correspondence, the proportion of travellers with diagnostic requests has also increased (Figure 1). Correlation between the number of requests, and IgG and IgM positive samples per year was very high (r_s = 0.82 to 0.99 and P <0.01). The proportion of patient samples testing positive for DENV IgG or IgM antibodies fluctuated, with peaks in 2005 and in 2009/2010. IgG and IgM proportions show a reasonably strong correlation (r_s = 0.70 and P <0.01). When studying trends in more detail, the increase relative to the previous year of IgG positives compared to IgM was higher in 2005 than in 2009, which indicates some independence between IgG and IgM proportions. A third peak was observed in 2002 but this is less clear as the total number of diagnostic requests was too low to draw conclusions based on this.

Trends in travel history by region

The 3,751 (42%) patients that had information on travel history provided with their diagnostic requests were subdivided into the geographical regions Africa, Americas, Europe and Asia-Oceania. For 263 patients, travel history was stated as 'Tropics' (228), 'No' (12) or 'Yes' (23) and these could not be assigned into a geographical category. Patients (n = 219) that had travelled to more than one region were included multiple times in the subdivision. Most patients traveling outside of Europe had travelled to Asia-Oceania followed by the Americas and then Africa (Table 2). The prevalence of DENV IgM and IgG positive patients differed significantly for travellers from different regions (Table 1, P<0.01). Only 29% (79) of the samples tested with PCR were accompanied by travel information (Table 2).

Trends in travel history by country

In total 131 different countries were listed in the travel history of patients. The top five travel destinations were Indonesia, Thailand, Surinam, India and the combination of the former and current Dutch Caribbean Islands, i.e. Bonaire, St Eustatius and Saba (BES islands), St Maarten, Aruba and Curacao (2000 patients, 53%). Travellers from Surinam were most frequently DENV IgG positive (Figure 2 and 3A). The Dutch Caribbean islands were the travel destination associated with the highest number of patients with DENV IgM positive tests (129 patients). Results are plotted for all countries that had been listed for at least 50 persons (Supplement figure 3A) or 20 persons (Supplement figure 3B) as travel destination.

The proportion of DENV positive patients differed greatly between travel destinations. Large differences were observed in percentage IgM positives for patients returning from different countries. This is partly explained by the low number of diagnostic requests for travellers to

these countries (Supplement figures 3A&B). Per top five travel destinations only one or two patients were PCR positive, but did show a higher than average number of positive samples compared to other destinations (20%).

Trends in DENV IgM positive results by country compared to WHO DENV notifications

Data representing the number of Dutch patients with DENV positive tests was combined with data representing the WHO reported DENV cases per country for the pooled data from patients returning from the top five travel destinations.

WHO notifications preceded the trends in DENV diagnoses from the pooled dataset by one year (data not shown). When analyzing trends by country, however, clear differences were observed. For the Dutch Caribbean islands, Suriname and Thailand, the diagnostic data and the number of WHO reported cases show a high correlation, although the timing differed.

Figure 1: Trends in diagnostic requests for evaluation of patients with suspected dengue virus infection from 2000 through May 2011(*) in The Netherlands in absolute counts (bars, first Y axis] and in proportions (lines, second Y axis)

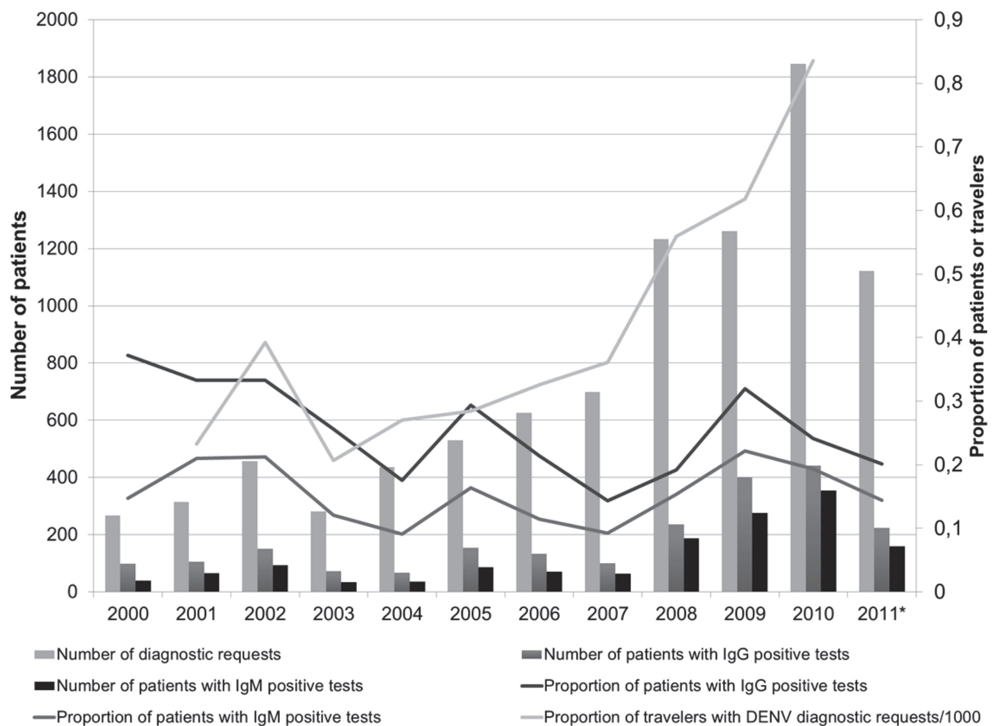


Table 1: Information provided with diagnostic requests per patient population for 8942 patients

Parameter	Percentage available
Sex	98%
Birth date	99,9%
Clinical history	75%
Travel history	42%
Sex, Age, Clinical, Travel history	38%
Vaccination history	0,5%
Clinical, Travel, Sex, Age, Vaccination	0,2%
Total patients	8942

Table 2: Reported travel destinations by region for returning travelers with suspected DENV clinical disease, and proportion with positive IgM serology, IgG serology, or both

Travel destination	Number of patients	% Requests	% IgG	% IgM	% IgG and IgM
Africa	643	17	11	5	3
Americas	1283	34	34	20	15
Asia-Oceania	1744	46	21	16	10
Europe	37	1	8	0	0
P-value			*<0,01*	*<0,01*	*<0,01*

The data from Thailand corresponded almost perfectly with the WHO data. For the Caribbean islands, the diagnostic trends preceded the increase seen in the WHO reporting by one to two years. For the data from travellers to Surinam, the increase in the diagnostic trend line from 2008 onwards was not seen in WHO notifications (Figure 2). For Indonesia, the diagnostic IgM trend showed some dependency with the WHO data but seemed to lag behind by one year in 2004-2005.

Finally, the proportion of IgM positive samples for India did not show any correlation with the WHO reported cases or the proportion of travellers with diagnostic requests. On the other hand the proportion of requests did show a correlation with the WHO data. All these observed correlations (r_s = 0.7 to 0.9) were significant (P<0.01).

Estimation of the proportion of DENV cases in travellers, 2007 and 2010

As travellers may be seen as a source of introduction of arboviral infections, we tried to use the available data to provide an estimate range for the possible number of dengue cases during one year, as described in the methods section. By this approach, the lower estimate, assuming that all clinical DENV imported cases would be detected, was 0.02% for 2007 and 0.06 % for 2010 of travellers to tropical or subtropical regions. The higher estimate, made based on the assumption that the current diagnostic requests reflect a fraction of all febrile travellers, was 3.7% for 2007 and 7.6% for 2010 of travellers to tropical or subtropical regions. We did not perform further calculations using the number of viremic travellers because of the low number of travellers tested by RT-PCR.

Discussion

Completeness of diagnostic data

We explored the potential use of diagnostic request data for systematic monitoring of trends in arboviral disease in Dutch travellers around the world, using DENV diagnostic data as a model [15]. We received information on 10,540 diagnostic samples from 8,942 patients.

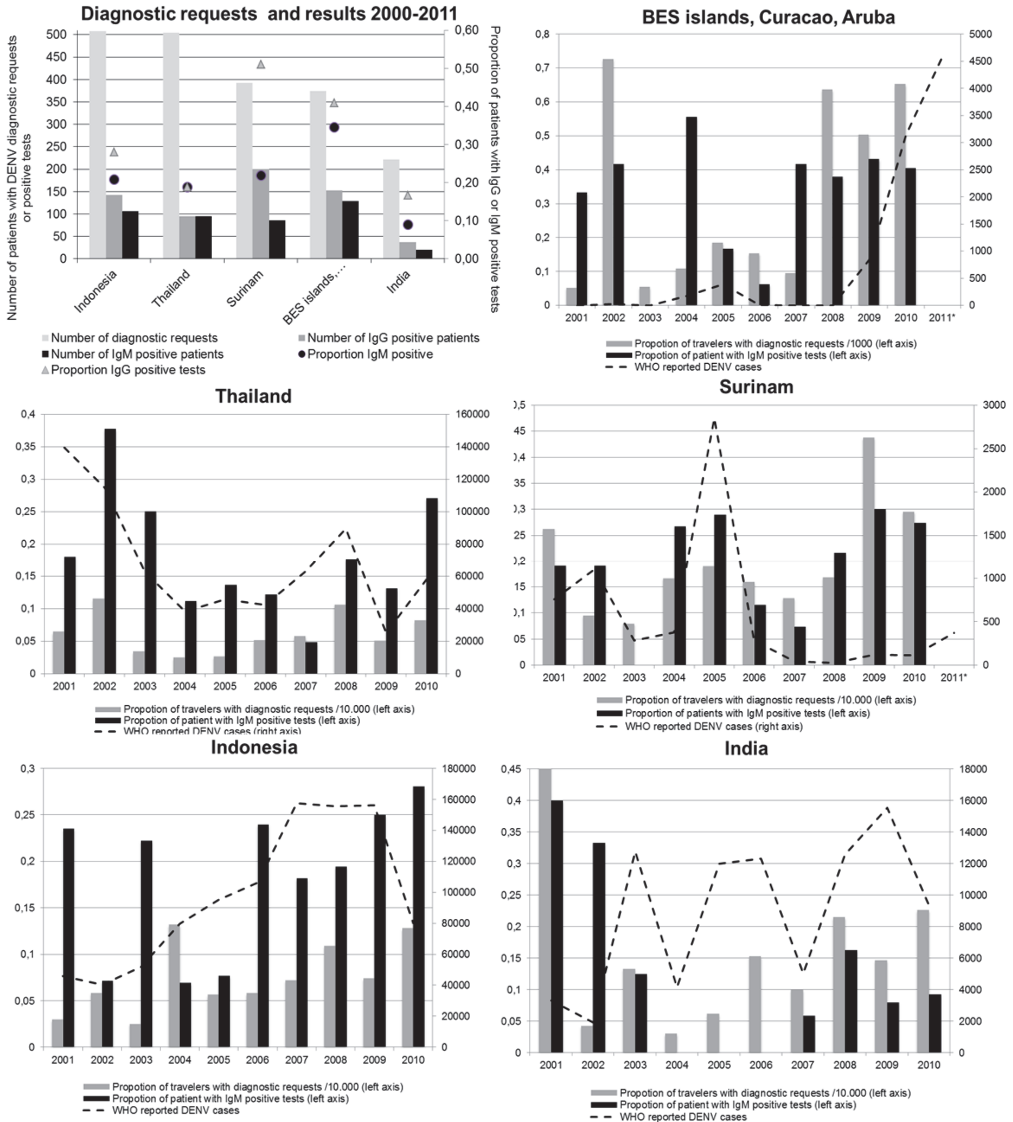
Only 42% of the diagnostic requests provided information on travel history thereby limiting the usable amount of data to a large extent. Because of such a large amount of requests this loss of usable data seemed to be partially compensated as analysis still provided results.

The literally complete lack of vaccination background data is of concern, as this information is essential for interpretation of serological diagnostic results of patients. The envelope protein of flavivirus is the main target for immune response [25-27]. Many common epitopes on the envelope protein cause cross-reactivity between flaviviruses in diagnostic tests [18, 28, 29]. Recent vaccination could cause cross-reactive antibody titers against other flaviviruses, mainly for IgG antibodies [16, 19, 30]. Although this factor has been taken into account when validating the cut-offs per laboratory for travellers to countries where YFV vaccination is compulsory (parts of South America) or highly recommended (Central Africa), this possible confounding factor could not be excluded when evaluating the IgG test results [16].

We tried to reduce the influence of this possible confounder by focusing further analysis on IgM data. This lack of information should be further investigated to identify probable causes along the diagnostic request chain, for example (1) shortage of time by physicians to complete request forms, (2) the lack in understanding of how vital this information is for interpretation or (3) the lack in knowledge of the usability of this data of surveillance. The use of digital request forms with compulsory fields might prove beneficial for improving completeness of diagnostic data.

Lack of additional information on travel times of individual patients could be compensated by extending the national information used on travel destinations into seasons and link these to the diagnostic trends per month. This approach can only be used when sufficient diagnostic requests are available, reflecting travel behaviour of the patient population that is subject to national preferences.

Figure 2: Trends in number of patients with requests and IgM positive tests (bars, first Y axis), WHO reported DENV cases and number of travellers (lines, second Y axis) per country from 2001 through May 2011(*)



Potentially combining information on an international level might provide a better spread in data per country and strengthen the data per visited country. This data could provide laboratory and background parameters for surveillance supplementary to what is already done through the Geosentinel system [6, 7].

Trends in diagnostic requests and results

The low number of requests for PCR is also of concern, especially because of the high percentage of PCR positive samples in both IgM positive and negative samples. In the Netherlands diagnostic samples may only be tested based on what the physician specifically requests. The lack in PCR requests is therefore probably based on the assumption that travellers are frequently not viremic anymore on time diagnosis. The number of PCR positive samples is therefore so low (one or two per destination] that no surveillance on travel destination is possible, but show that potentially a large number of viremic travellers return to the Netherlands annually.

There was a clear increase in the DENV requests and IgM positive patients over recent years (Figure 1), similar to what was observed in other countries across the EU like Germany and the United Kingdom [31-33]. The proportion of travellers with diagnostic requests has increased (Figure 1). This may reflect true increase in DENV prevalence or more awareness of DENV risk among Dutch physicians.

Trends in travel history per region

Although most diagnostic requests involved patients returning from Asia, the most positive samples came from the Americas, possibly for the reasons given above, whereas DENV was rarely diagnosed in travellers returning from Africa (Table 1). This is interesting as DENV is endemic in a large part of Africa. Possibly physician awareness might play a role in requesting dengue testing in travellers to Africa.

Trends in travel history per country

Three of the five top travel destinations for Dutch travellers have a colonial past with the Netherlands and therefore the high number of travellers in part are explained by citizens visiting friends and relatives. Such specific groups of travellers may bias results for two reasons: the local population might have more up to date knowledge of currently circulating diseases and might inform the visitors of possible DENV when they get ill.

Secondly, the higher contribution of frequent visitors may influence the proportion of IgG-only positive patients as can be seen for Surinam where the percentage of IgG positive patients was higher than for other travel destinations (Figure 2). However, Surinam is also a yellow fever endemic country where vaccination is recommended. The high IgG proportion could therefore also be due to yellow fever vaccination cross-reactivity [16, 19, 30].

From this data alone, it is difficult to distinguish between these possibilities because of a small percentage of patients with vaccination history. The high frequency of IgG positive samples also emphasizes the need for a convalescent sample for identification of a recent

infection. This does support focusing trend monitoring more on IgM results and less on IgG until information on vaccination history is provided more regularly.

Trends in DENV IgM positive results by country compared to WHO DENV notifications

WHO notification data on the Caribbean, Central America and the northern part of South America indicate large DENV outbreaks in 2007, 2009, 2010 and high DENV activity in 2008 [34]. This corresponds to our diagnostic findings for Surinam. It seems implausible that all Surinam surrounding countries would experience outbreaks and Surinam would remain unaffected. Possibly, reporting of DENV cases to the WHO was affected for some reason during this period. Monitoring based on diagnostic results with WHO notification therefore has a clear added value.

The diagnostic results for travellers from Indonesia and India show less correlation. Both these destinations are less popular than the previously mentioned three. Dutch travellers focus their travels mainly on Borneo, Bali and Java islands making them less representative for all Indonesia. Focusing outbreak information on only these three islands may prove to be more accurate in relation to travellers.

Occasionally, a deviation from the trend indicated presence of high numbers of patients with dengue-like symptoms, for instance in the data for India for 2005 and 2006. This may indicate an outbreak of another disease with symptoms comparable to DENV (hence the DENV diagnostic request) but that causes no cross-reactivity (thus probably not a flavivirus). Outbreak reports suggest a possible increase in chikungunya infections in this area from 2005 onwards that might influence the increase in probable DENV clinical cases reported [35, 36].

The data from the WHO is dependent on reporting of local health institutes and laboratories, each using different diagnostic methods, and not always based on diagnostic confirmation. For some regions the reliance on clinical case notifications might influence DENV trends of clinical syndromes overlap, as for instance is the case for Chikungunya virus infection.

Estimation of the proportion of DENV cases in travellers, between 2007 and 2010

The estimated proportion of dengue cases in travellers was found to be between 0.02%-3.7% and 0.06%-7.6% of all travels to DENV risk countries in 2007 and 2010. This estimation seems to be in accordance with earlier prospective studies by Cobelens et al. in 1991-1992 who found 1.7-5.1% incidence rate of dengue for travellers to high risk countries in Asia, and Baaten et al. in 2006-2007 who found that 1.2% of travellers to the tropics seroconverted [33, 37]. The calculation of the number of infected travellers showed a 100-fold difference between lower and higher estimates, but indicated that the chances of DENV importation into the Netherlands due to viremic travellers are potentially high.

This information does provide some overview of potential risks. This may become relevant in case of mosquito invasions, as were observed in Italy with the outbreak of chikungunya due to introduction by a traveller and subsequent transmission through mosquitoes that were competent for transmission of alphaviruses [38]. Tilston et al. used the estimated number of

viremic travellers flying into Europe from known chikungyna outbreaks areas to identify high risk regions in Europe for chikungunya outbreaks. They proposed an enhanced traveller-based surveillance in parts of Europe supported by this estimation [39]. Our data could be a further addition to this surveillance approach.

Our study also has weaknesses: the dataset is biased because (a) recruitment is dependent on a patient developing symptoms severe enough to seek out medical care, (b) diagnostic requests are based on awareness of physicians, (c) completeness of data provision by physicians influences the usefulness for surveillance, and (d) travel behaviour is specific to the Netherlands.

Conclusion

We showed that trends in diagnostic requests and results can be identified and correspond partially to the WHO DENV reports on DENV outbreaks per country for the three top popular travel destinations of Dutch travellers. Occasional discrepancies were seen that could be explained either by underreporting of data to the WHO, or under-diagnosis of DENV as a cause of illness in travellers. The data offers a wide range of additional parameters that can be used as a source of information not only on current possible outbreaks, but also changes in awareness as well as high or low risk countries or populations.
Secondly, this approach may also provide information on disease outbreaks with other pathogens causing diseases that are similar to DENV clinically. Currently, the number of PCR tested samples are too low and provides insufficient data for trend analysis based on travel destination, but shows that potentially a large number of viremic travellers return to the Netherlands annually.

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Ethical approval: In accordance with the Dutch law on medical research (WMO), article 1.

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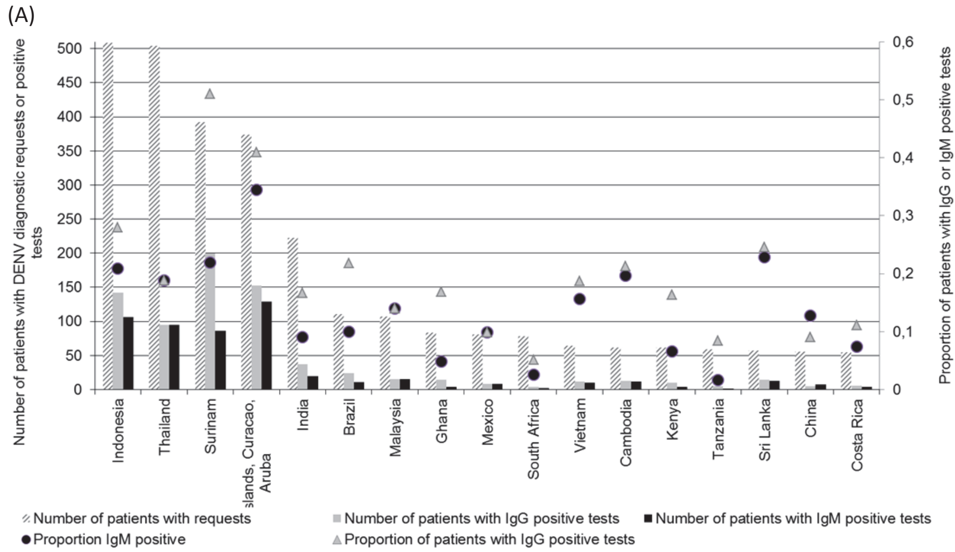
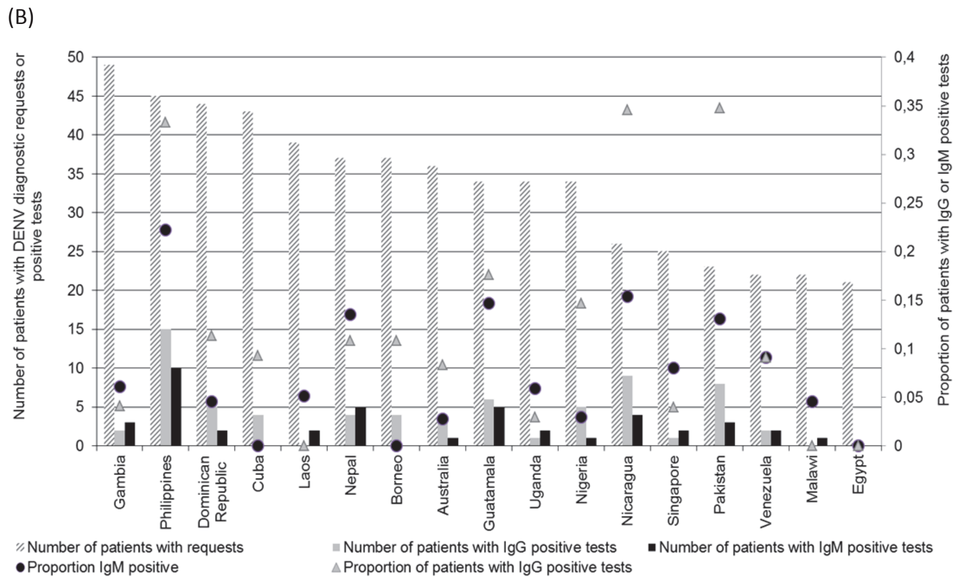
Supplement figure 3: Number of requests for DENV diagnostics in returning travellers, and test results by country, listed for A) the most common travel destinations (50 or more patients with diagnostic requests between 2000-2011), and B) less common travel destinations (50 or less patients). The number of requests, number IgM and IgG positive are indicated in bars (Y axis), the proportion IgM and IgG positive samples are indicated by the dark circle and grey triangle, respectively (second Y axis).

(A)



(B)



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Come fly with me:
review of clinically important arboviruses for global travelers

Cleton N., Koopmans M., Reimerink J., Godeke GJ. and Reusken C.

Abstract

Western tourists are increasingly traveling to exotic locations often located in tropical or subtropical regions of the world. The magnitude of international travel and the constantly changing dynamics of arbovirus diseases across the globe demand up-to-date information about arbovirus threats to travelers and the countries they visit. In this review, the current knowledge on arbovirus threats to global travelers is summarized and prioritized per region. Based on most common clinical syndromes, currently known arboviruses can be grouped to develop diagnostic algorithms to support decision-making in diagnostics. This review systematically combines and structures the current knowledge on medically important travel-related arboviruses and illustrates the necessity of a detailed patient history (travel history, symptoms experienced, vaccination history, engaged activities, tick or mosquito bite and use of repellent and onset of symptoms), to guide the diagnosis.

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Introduction

Globally the number of travelers has risen from 450 million in 1990 to nearly 950 million in 2010.[1] Western tourists are increasingly traveling to more exotic medically high-risk locations in developing countries or upcoming economies, like China and India, often located in tropical or subtropical regions of the world.[1] 5-10% of travelers report to a medical care taker after travel.[2] Consequently, doctors are increasingly confronted with travel-related diseases, stressing the need for awareness within the medical profession and general population.

The differential diagnosis of fever in travelers is long, including cosmopolitan as well as more exotic infections.[3] The most important syndrome is diarrhea, followed by undifferentiated fever and dermatological problems depending on travel-destination.[2] Although malaria remains the most important cause for systemic febrile disease in travelers, arbovirus infections belong in the differential diagnosis.[3] This is emphasized by the rise in the proportion of travelers being diagnosed with exotic arbovirus infections like chikungunya virus and dengue virus, with dengue currently being the second most important cause for febrile disease in travelers.[4-7]

The magnitude of international travel and the constantly changing dynamics of arbovirus diseases across the globe demand up-to-date information about current arbovirus threats to travelers and the countries they visit.

Establishing a differential diagnosis requires up-to-date knowledge based on evolution of the patient's symptoms, travel history, specific background information on possible exposures and test results. This review focuses on all medically important arboviruses, to facilitate clinicians and clinical laboratories in their differential diagnosis. It summarizes current literature on risk of arbovirus infection in global travelers, and prioritizes them per region.

Background on arboviruses (Tables 1 and 2)

Arboviruses use arthropod vectors as their main transmission route and are therefore defined as ARthropod-BOrne viruses. Mosquitoes, ticks, midges and sandflies are known virus-transmitting arthropods. The majority of arboviruses belong to the *Flaviviridae, Bunyaviridae* or *Togaviridae* families, but a small number are member of the *Reoviridae,* and *Orthomyxoviridae* families (Tables 1 and 2).[8, 9, 10]

Of the over 545 suspected arbovirus species more than 150 are documented to cause disease in humans, and the majority are zoonotic, They are sustained in a transmission cycle between arthropods as vectors and vertebrate animal reservoirs as main amplifying hosts (Table 1). Humans are infected in spill-over events and are often dead-end hosts, as they do not develop the high viremias needed to infect arthropods.[8, 11]

Only a few viruses like yellow fever, chikungunya and dengue virus have expanded their host range to include humans as an amplifying host. They can lead to mosquito-borne disease outbreaks, often in urban settings, without the need of an animal reservoir. This urban transmission cycle in part explains the 'success' of these viruses.[12]

Based on the pattern of occurrence the individual viruses in Table 1 were labeled as endemic (reflecting stable presence in a reservoir), sporadic (reflecting isolated infections), or epidemic (reflecting occurrence during seasons with increased disease activity or outbreaks). Large epidemics can occur for example because of climate variations, like extraordinary rainfall or movement of large populations or viruses into new areas.[13]
Arboviruses may also be transmitted through blood from viremic patients, which is a particular concern for the blood supply in endemic areas and when taking care of patients with hemorrhagic fever.[14-17] Cases of human-to-human transmission of West Nile virus through blood transfusions and organ transplantation have been reported,[15, 18-20] but all arboviruses that produce viremia in humans are thought to be a potential risk (Table 2).[21, 22]

Bunyaviridae.
The genera *Orthobunya* , *Phlebo* and *Nairovirus* within the *Bunyaviridae* family, contain human arboviruses (Tables 1 and 2).[23] *Orthobunyaviruses* use mosquitoes and/or midges as their main vectors.[24] This genus is divided into 18 serogroups, based on cross titrations in haemaglutination inhibition (HI) assays and neutralization assays (NT), and correlating to main vector preferences. Many hold viruses that have been reported to cause disease in humans.[25-27] However, the most clinically important travel-related viruses are found in only two serogroups, the California encephalitis serogroup and the Simbu serogroup.
Nairoviruses use ticks as main vector and comprise 7 serogroups.[28] Only Crimean-Congo hemorrhagic fever virus is considered to be of clinical importance to travelers.[24, 29]
The *Phlebovirus* genus contains the phlebotomus fever serogroup (sandfly-borne viruses). The most clinically important are Toscana virus, which is transmitted by sandflies, and Rift Valley fever virus, which is transmitted by mosquitoes.[23] This example also illustrates that taxonomy based on vector preference, and vice versa, may not be consistent.
The clinically important viruses are found across serogroups and in distinct geographical areas, reducing the problems in diagnostic test interpretation due to cross-reactivity when the travel destination is reported (Table 3 and Maps 1-3).

Flaviviridae.
The family *Flaviviridae* is divided into 3 genera. Only the *flavivirus* genus holds arboviruses, some of which are the most clinically important arboviruses world-wide, like dengue, yellow fever and West Nile virus (Tables 1 and 2).[30, 31] The human flaviviruses are divided into nine serogroups.[32, 33] Five of these contain medically important arboviruses (Tables 1 and 2). Depending on the serological assays used, cross-reactivity between serogroups may complicate interpretation of diagnostic assays. Two main flavivirus transmission routes are recognized: tick-borne and mosquito-borne.[33, 34] Inquiring about a history of tick or mosquito-bite during patient evaluation can help focus the differential diagnoses.

Reoviridae

Three genera of the *Reoviridae* contain arboviruses but only two are considered potentially important travel-related viruses, i.e. Banna virus in the *Seadornvirus* genus and Colorado tick fever virus in the *Coltivirus* genus (Tables 1 and 2).[35-38] These two viruses are found on opposite sides of the world (resp. Old and New World), use different vectors (resp. mosquitoes and ticks) and are not cross-reactive in serology as they differ in genus (Map 1).

Togaviridae

Arboviruses are found in the *Alphavirus* genus of the *Togaviridae* family.[39] The *Alphavirus* genus is divided into seven serogroups of which six contain clinically important viruses for travelers (Tables 1 and 2).[39, 40] Mosquitoes are their main vector.[41] About 50% of the alphaviruses cause disease in humans.[39] Because there is a clear division in New and Old World alphaviruses, the use of travel history of patients can aid substantially in focusing the differential diagnosis (Table 3). Some Old and New World viruses are found in the same cross-reacting groups, like chikungunya and mayaro virus. Travel history can facilitate serology test interpretation by excluding cross-reactive viruses based on their geographic distribution (Maps 1-3).

Clinical manifestations (Table 2)

Clinical symptoms of arbovirus infections in humans can be divided into four main clinical syndromes defined as mild or severe febrile illness (FD), rash and arthralgia (AR), neurological syndrome (NS) and hemorrhagic syndrome (HS).[8, 42] Other symptoms such as hepatitis, bronchopneumonia and conjunctivitis are also reported.[42, 43] FD generally presents as flu-like symptoms such as fever, headache, retro-orbital pain and myalgia. NS can manifest as myelitis, meningitis and/or encephalitis, with behavioral changes, paralysis, paresis, convulsions and coordination problems.[40, 44, 45] AR manifests as exanthema or maculopapular rash, polyarthralgia and polyarthritis.[46-49] HS can clinically present as ptechiae, spontaneous or persistent bleeding and shock combined with a severely low platelet count, increased PT/PTT and liver enzymes.[28, 50] Syndromes largely overlap, making a diagnosis based on clinical symptoms alone impossible (Table 2 and Map 1-3).

Bunyaviridae

The clinical infection rate for bunyaviruses is less well defined, but the majority of infections are thought to be asymptomatic. Exceptions are Crimean-Congo hemorrhagic fever and Oropouche virus, which cause clinical disease in respectively 25% and 30-60% of the infected persons.[28, 51-55] Bunyavirus infections generally present as FD but some can progress to more severe forms of HS (Crimean-Congo hemorrhagic fever and Rift Valley fever virus) and NS (La cross virus and Toscana virus) (Table 2).[28, 55] The highest case fatality rates are seen in cases that develop HS or NS, reaching 30% for complicated Crimean-Congo hemorrhagic fever infections.[28, 50, 56] A number of endemic viruses found in Sub-Saharan Africa and South America present as FD with or without AR.[57-64] This makes them

clinically indistinguishable from other common infections like malaria, yellow fever, dengue and chikungunya.

Flaviviridae

On average less than 10% of flavivirus infections are thought to result in clinical symptoms, with complications in an even smaller proportion of cases. If patients develop life-threatening syndromes like HS or NS, case fatality rates may be as high as 30%.[3, 34, 35, 37, 44, 65-70] Yellow fever virus is the exception as up to 50% of infected persons develop clinical symptoms.[71, 72] The Japanese encephalitis group has FD and NS as its main clinical syndromes. The dengue virus group presents as FD, AR and in severe cases shock or hemorrhagic fever. It is important to note that the number of patients with severe forms of dengue seems to be on the rise due to an increase in secondary infections with a heterologous serotype.[66, 73-75] Within the tick-borne flaviviruses, the Asian-Middle East viruses (e.g. Alkhurma virus) are known to cause HS while the European and American viruses (e.g. tick-born encephalitis) present as NS.[36, 37, 76-78]

Reoviridae

Both Banna virus (BANV) and Colorado tick-borne encephalitis (CTFV) present with FD and NS.[35, 36, 38, 79] CTFV has a very specific geographical distribution and the number of confirmed cases is low.[35, 36] The geography of BANV is unclear and is thought to correspond with Japanese encephalitis virus (JEV). BANV is clinically indistinguishable from JEV.[38] As *Reoviridae* serologically do not cross-react with other families, it is important to test for BANV too if JEV is suspected.

Togaviridae

Togaviruses reportedly have a high clinical attack rate with 50-85% of infections resulting in clinical manifestation.[46-48, 80, 81] Sindbis virus, Western equine encephalitis virus and Eastern equine encephalitis virus are exceptions with 0,1%-5% symptomatic infections. Equine encephalitis virus infected patients develop FD followed by NS.[39, 45, 82] For all other viruses FD is accompanied by AR and therefore clinically difficult to distinguish from dengue virus infections. Typical for AR caused by alphaviruses is the prolonged symptoms of arthritis and arthralgia that can persist from months to years after infection.[83]

Treatment and prevention (Table 2)

Depending on the etiology, different complications can occur when the disease develops beyond the febrile stage. There are no specific effective antiviral treatments for any of the arboviruses, and thus only supportive care like fluid and electrolyte management are available.[34] Early recognition and confirmation of the disease makes it possible to adapt supportive care pre-emptively to a variety of specific complications, thus increasing survival rate substantially.

The hallmark prevention of arbovirus infection in travelers consists of the use of repellents, fine-mesh bed nets treated with repellent and behavioral aspects such as regular checks for possible ticks. Vaccines are available only for a few flaviviruses (Table 2).
Currently, there are two JEV vaccines on the market based on inactivated virus; Ixiaro (Intercell Biomedical, Livingston, UK) and JE-vaccin (GCVC, Korea). For KFDV, a formalin-inactivated vaccine is registered only in India and is currently produced by Institute of Animal Health and Veterinary Biological Laboratory in Bangalore. For TBEV a number of vaccines from different producers in Europe, Russia and China are available that are based on inactivated viruses.[84, 85] A number of yellow fever attenuated live-attenuated strain 17D vaccines are available from different producers around the world.[86] Vaccines for other viruses like DENV, CHIKV and RVFV are at different stages of research.

Assessing risk for travelers (Table 3 and Map 1-3)

Some general factors can be used to prioritize infection risk if specific risk estimation is lacking: a) (sub)urban circulation of the virus, b) known endemic disease, c) recorded cases of infections in travelers.[87-90]
Obviously, this can only be done if some information is available, which may be challenging in regions with limited surveillance. There are numerous arboviruses of which the potential human health impact is poorly characterized. Despite these unknowns we have summarized the available information for the most important travel associated arboviruses, grouped them in broad probability classes (Table 3) and visually summarized their geographical distribution in relation to symptoms and taxonomy in a number of maps (Maps 1-3).
Specific risk estimates are available for a few arboviruses. RRV infections in travelers have been documented regularly, and large outbreaks in the South Pacific are thought to be due to viremic travelers.[91-94] New Zealand estimated that annually 100 viremic travelers return home from Australia.[48, 92]
DENV is by far the most diagnosed arbovirus in travelers.[69, 95-99] Of the travelers presenting with febrile disease, 3-8% have serological evidence of current dengue fever.[3, 7, 95, 100, 101] Serological surveys suggested that the actual infection risk in travelers might be significantly higher and that dengue is underdiagnosed.[7, 102]
In Asia, one on the most common viral causes of encephalitis is Japanese encephalitis virus (JEV).[44] However, the risk to travelers is considered low and has been estimated at 1 in 1 million for urban travelers and 1 in 20,000 per week of stay for travelers to the rural areas for longer periods of time.[103] YFV is the most common arbovirus in Africa.[71, 72] Risk for unvaccinated travelers is estimated at 1:280 in Africa and 1:2800 in South America in epidemic areas.[72] In endemic areas, risk of infection is about 20-fold lower. Tick-borne encephalitis virus (TBEV) is one of the most frequently diagnosed European arboviruses in travelers. Risk of clinical infection in travelers to Austria, one of the main endemic countries, is estimated at 1 in 10,000 people per month stay.[104, 105]

Diagnosis of arbovirus infections

Laboratory diagnosis of arbovirus infections is based on the detection of the virus or viral antigens, or the detection of antibodies. Serum taken at multiple time points is the preferred specimen. In case of central neural system involvement, a cerebrospinal fluid (CSF) sample should be added. The preferred diagnostic method depends on the duration of the incubation period, and associated with that, the specific pattern of viremia and antibody development.

Virus and viral antigen detection is the most specific method to confirm arbovirus infection. Methods available are polymerase chain reaction (RT-PCR) and virus isolation (VI) from serum, tissue or CSF. In addition, for flavivirus infections, kits for detection of the infection-associated NS1 protein are available in a number of different formats.[106, 107] Sandfly fever, West Nile and Eastern equine encephalitis virus, for example, produce very short and low viremias and symptoms typically develop after this viremic phase, explaining the relatively low sensitivity of PCR-based diagnosis.[108, 109] In travelers, the acute phase of the disease is frequently missed because patients often visit a health care worker after returning home when symptoms persist or worsen beyond the febrile stage.

Serology is therefore the most commonly used diagnostic method for arbovirus infections. Commercially available and/or in-house IgG/IgM enzyme-linked immunosorbent assay (ELISA) kits, haemaglutination inhibition (HI), immunofluorescence assay (IFA) and neutralization (VNT) assays are used.[106] Typically, IgM antibodies develop within a few days after infection and can generally be detected up to three months after infection. IgG antibodies develop within days after IgM and can be detected months to years after initial infection. IgM antibodies can persist for longer periods and complicate diagnostics.[110, 111] IgM and IgG antibodies in serum can be used for the detection of past and current infections if paired sera are collected with a 10-14 days interval. A seroconversion or a 4-fold or greater increase in titer is required for confirmation.[106] The detection of IgM antibodies in the CSF also implies recent infection.

Interpretation of the results requires knowledge about the specific method used, and on patient background, for instance travel- and vaccination history. Infections or vaccinations with flaviviruses may trigger cross-reactive antibodies, when using ELISA or IFA assays for diagnosis.[112, 113] VNT can be used if further confirmation of a specific viral infection is required.

Validated diagnostic tests are available for the most common viruses, but not for all. Here, referral to a specialized arbovirus laboratory may be needed. The European network for diagnostics of imported viral diseases (ENIVD) provides a network to identify all available special diagnostics in specialized laboratories across Europe.[114]

Table 1: Summary of taxonomy and essential ecology of medically important travel-related arboviruses

Family *Genus*	Serogroup	Virus	Abr.	Vector	Host
Bunyaviridae					
Nairovirus	Crimean-Congo hemorrhagic fever	Crimean-Congo Hemorrhagic fever virus	CCHFV	Tick	Domestic and wild animals, birds, small mammals
Orthobunyavirus	Bwamba	Bwamba virus	BWAV	Mosquito	Unknown
	Bunyamwera	Bunyamwera	BUNV	Mosquito	Possibly rodents
		Ilesha virus	ILEV	Mosquito	Unknown
		Ngari virus	NRIV	Mosquito	Unknown
	California encephalitis	La Cross virus	LCV	Mosquito	Small mammals
		Guaroa virus	GROV	Mosquito	Unknown
		Tahyna virus	TAHV	Mosquito	Hares, rabbits, hedgehogs, small mammals
	Simbu	Oropouche virus	OROV	Midge	Humans, Sloths (maybe primates, birds)
	Ungrouped viruses	Tataguine virus	TATV	Mosquito	Unknown
Phlebovirus	Phlebovirus fever	Toscana virus	TOSV	Sandfly	Humans, bats
		Sandfly fever	SFV	Sandfly	Human, rodents
		Rift Valley fever virus	RVFV	Mosquito	Rodents, bats, cattle
Flaviviridea					
Flavivirus	Dengue virus	Dengue virus	DENV	Mosquito	Primates, humans
	Japanese encephalitis	Japanese encephalitis viru	JEV	Mosquito	Ardeid birds, pigs
		West Nile virus	WNV	Mosquito	Birds
		St. Louis encephalitis virus	SLEV	Mosquito	Birds
		Murray Valley virus	MVEV	Mosquito	Ardeid birds
	Mammalian tick-born virus group I	Kyasanur Forest disease v	KFDV	Tick	Small mammals, humans
		Alkhurma hemorrhagic fever	AHFV	Tick	Small mammals
		Tick-borne encephalitis	TBEV	Tick	Small mammals, birds
	Ntaya virus	Ilheus virus	ILHV	Mosquito	Birds
	Spondweni group	Zika virus	ZIKV	Mosquito	Primates, humans
	Yellow fever	Yellow fever virus	YFV	Mosquito	Primates, humans
Reoviridae					
Coltivirus	Colorado tick fever	Colorado Tick fever	CTFV	Tick	Small mammals
Seadornavirus	Bunna	Banna virus	BANV	Mosquito	Unknown
Togaviridae					
Alphavirus	Barmah Forest	Barmah Forest virus	BFV	Mosquito	Wild birds, marsupials
	Eastern eq. encephalitis	Eastern equine encephalitis virus	EEEV	Mosquito	Aquatic birds, small mammals, marsupials
	Semliki forest	Chikungunya virus	CHIKV	Mosquito	Primates, humans
		Mayaro virus	MAYV	Mosquito	Primates birds, humans
		O'Nyong-nyong	ONNV	Mosquito	Primates, humans
		Ross River virus	RRV	Mosquito	Marsupials, mammals
	Western eq. encephalitis (sindbis like)	Sindbis virus	SINV	Mosquito	Birds
	Western eq. encephalitis (recombinants)	Western equine encephalitis virus	WEEV	Mosquito	Birds, small mammals
	Venezuelan eq. encephalitis	Venezuelan equine encephalitis virus	VEEV	Mosquito	Small mammals

* = Occurrence: En= Endemic, Ep = Epidemic, Sp = Sporadic

Table 1: Continued

Family *Genus*	Virus	Geographical distribution	Occur.*	Ref.
Bunyaviridae				
Nairovirus	Crimean-Congo Hemorrhagic fever virus	South-East and Eastern Europe, Africa, Asia	Sp	28, 56, 98, 117, 118
Orthobunyavirus	Bwamba virus	Sub-Saharan Africa	En	26, 119
	Bunyamwera	Sub-Saharan Africa	En	25, 119
	Ilesha virus	Sub-Saharan Africa	En	119, 120
	Ngari virus	Sub-Saharan Africa	En	121, 122
	La Cross virus	North America	En	52, 123
	Guaroa virus	Central and South America	En	57, 60
	Tahyna virus	Europe, Asia, Africa	En	53, 124, 125
	Oropouche virus	Central and South America	En	58, 60, 126
	Tataguine virus	Sub-Saharan Africa	En	64, 127
Phlebovirus	Toscana virus	Southern Europe	En	54, 55
	Sandfly fr	Southern Europe, Northern Africa, Asia	En	55
	Rift Valley fever virus	Africa, Western Asia	En and Ep	13, 128
Flaviviridea				
Flavivirus	Dengue virus	Asia, Africa, Americas	En and Ep	66-68
	Japanese encephalitis virus	South and South-East Asia, Oceania	En and Ep	129, 130
	West Nile virus	North and South America, South and Eastern Europe, South-East Asia, Oceania	En and Ep	131-133
	St. Louis encephalitis virus	Americas	En and Sp	134
	Murray Valley virus	Oceania	En	77, 135
	Kyasanur Forest disease	South-East and Western Asia	Ep	136, 137
	Alkhurma hemorrhagic fever virus	Western Asia	Ep	138
	Tick-borne encephalitis virus	Central, Northern and Eastern Europe, and Asia	En	37, 65
	Ilheus virus	Central and South America	En	31
	Zika virus	Asia, Africa, Americas	En and Ep	
	Yellow fever virus	Sub-Saharan Africa and South America	En and Ep	71
Reoviridae				
Coltivirus	Colorado Tick fever virus	North America	Sp	36, 139
Seadornavirus	Banna virus	Asia	En	38
Togaviridae				
Alphavirus	Barmah Forest virus	Australia	Ep and Sp	113, 140
	Eastern equ. encephalitis	Americas	Ep and Sp	40, 141
	Chikungunya virus	Africa and Asia	En and Ep	81, 142
	Mayaro virus	South America	En	80, 143
	O'Nyong-nyong	Sub-Saharan Africa	En and Ep	144, 145
	Ross River virus	Oceania	Ep	91, 140
	Sindbis virus	Northern Europe, Asia, Africa, Oceania	Ep	82, 127, 146
	Western eq. encephalitis	Americas	Sp	40, 147
	Venezuelan eq .encephalitis	Americas	En and Ep	148

Table 2: Summary of the reported information on health impact associated with arboviruses listed in Table 1

Family *Genus*	Virus	Symptoms*	Incubation period	Vaccine	Circ.**	Reported in travelers
Bunyaviridea						
Nariovirus	Crimean-Congo hemorrhagic fever	FD, HS, (NS)	1-3 (1-9)	Yes	R	Yes
Ortho-bunyavirus	Bwamba virus	FD, AR, (NS)	1-14	No	R	No
	Bunyamwera virus	FD, AR, (NS)	Unknown	No	R	No
	Guaroa virus	FD, AR	Unknown	No	R	No
	Ilesha virus	FD, AR (NS, HS)	Unknown	No	R (U)	Yes
	Ngari virus	FD, AR, HS	Unknown	No	R	No
	La cross virus	FD, NS	5-15	No	R	No
	Tahyna virus	FD, AR, (NS) conjunct bronchopn	3-7	No	U	No
	Oropouche virus	FD, AR, (NS)	4-8	No	R, U	No
	Tataguine virus	FD, AR	Unknown	No	R	No
Phlebovirus	Toscana virus	FD, NS, (AR)	2-14	No	R	Yes
	Sandfly fever	FD	2-14	No	R	Yes
	Rift valley fever virus	FD, HS, NS, hepatitis	1-7	No	R	Yes
Flavivirus						
Flavivirus	Dengue virus	FD, HS, AR, (NS)	4-7 (3-14)	No	R, U	Yes, 3-8% travelers with FD
	Japanese encephalitis virus	FD, NS	5-14	Yes	R, U	Yes, 1 in a million or 1 in 20.000
	West Nile virus	FD, NS, AR	3-5 (2-14)	No	R, U	Yes
	St. Louis encephalitis v	FD, NS	2-21	No	R, U	No
	Murray Valley virus	FD, NS	1-28	No	R	Yes
	Kyasanur Forest disease virus	FD, HS, conjuncti, pneumonia	3-8	Yes	R	No
	Alkhurma hemorrhagic fever	FD, HS	3-12	No	R	Yes
	Tick-borne encephalitis virus	FD, NS, (HS)	7-14	Yes	R	Yes, 1:10.000 man/month
	Ilheus virus	FD, NS	Unknown	No	R	No
	Zika virus	FD, AR, conjuctivitis	3-5 (2-14)	No	U	Yes
	Yellow fever virus	FD, HS, hepatitis	3-6	Yes	R, U	Yes, 1:280 Africa to 1:2800 SA
Reoviridea						
Coltivirus	Colorado tick fever	FD, NS, AR, HS	3-5 (0-20)	No	R	Yes
Seadronvirus	Banna virus	FD, AR, NS	Unknown	No	R	No
Togaviridea						
Alphaviruses	Barmah Forest	FD, AR	7-9 (5-2)	No	R, U	No
	Eastern eq. encephalitis	FD, NS	3-10	No	R	Yes
	Chikungunya virus	FD, AR, (HS, NS) conjunctivitis	3-7 (1-12)	No	R, U	Yes
	Mayaro virus	FD, AR, (HS)	6-12 (3-12)	No	R, U	Yes
	O'Nyong Nyong	FD, AR	>8	No	R, U	No
	Ross river virus	FD, AR, (HS)	7-9 (3-21)	No	R, U	Yes, 100 per year to New Zealand
	Sindbis virus	FD, AR	1-7	No	R	Yes
	Western eq. encephalitis	FD, NS	2-10	No	R	No
	Venezuelan eq. encephalitis	FD, NS	<1-5	No	R	No

Symptoms*: FD = Febrile disease, AR = Arthralgia and/or Rash, HS = hemorrhagic syndrome, NS = Neurological syndrome. Circ.** = Circulation: R = Rural, U = Urban

3

Table 2: continued

Virus	Seroprev.	info	CUD	H2HT	Ref.
Bunyaviridea					
Crimean-Congo hemorrhagic fever	High: 5-13%	Yes	Yes	Yes	14, 17, 29, 50, 149
Bwamba virus	High: 2,6-80%	Little	Yes	No	26, 27, 61
Bunyamwera virus	High: up to 100%	Little	Yes	No	25, 121
Guaroa virus	High: 13–18%	Little	Yes	No	57, 60
Ilesha virus	High: 38-54%	Little	Yes	No	62, 120
Ngari virus	Unknown: in a recent RVFV outbreak 27% of infections turnout to be NRIV	Little	Yes	No	121, 122
La cross virus	High: 2-13%	Yes	Yes	No	52, 123, 150-152
Tahyna virus	High: 60-80%	Little	Yes	No	53, 124, 125, 153
Oropouche virus	High: 15-60%	Yes	Yes	No	51, 58, 60
Tataguine virus	High: one of the five most isolated viruses in West Africa in humans	Little	Yes	No	64, 127
Toscana virus	High: 5-51%	Yes	No	No	54, 154
Sandfly fever	High: 3-36%	Yes	No	No	55, 155
Rift valley fever virus	High :2-14%	Yes	No	Yes	13, 50, 128, 156-159
Flavivirus					
Dengue virus	High: most diagnosed arbovirus disease world-wide	Yes	Yes	Yes	5-7, 69, 160-162
Japanese encephalitis virus	High: >45.000 cases annually world-wide	Yes	Yes	No	103, 163
West Nile virus	High: One of the most common arbovirus diseases world-wide	Yes	No	Yes	19, 20, 131, 164
St. Louis encephalitis virus	High: 3-13%	Yes	Yes	No	134, 152
Murray Valley virus	High: up to 40%	Yes	No	No	135, 165-167
Kyasanur Forest disease virus	Unknown: local monkey population 18-50% positive	Yes	No	No	136, 137
Alkhurma hemorrhagic fever	Low: 1,3%	Yes	No	No	138, 168, 169
Tick-borne encephalitis virus	High: incidence rate of 0.1 to >5: 100.000 in endemic countries	Yes	Yes	Yes	170-173
Ilheus virus	High :3,4-26%	Little	Yes	No	174, 175
Zika virus	Unknown	Little	Yes	Yes	
Yellow fever virus	High: 200.000 cases annually world-wide	Yes	No	Yes	29, 50, 176-179
Reoviridea					
Colorado tick fever virus	Unknown: 200-400 reported cases annually	Yes	Yes	Yes	36, 180, 181
Banna virus	Unknown	Little	Yes	No	38
Togaviridea					
Barmah Forest virus	High: incidence rate of 4 to 40:100.000 annually. 500-800 reported cases annually	Yes	No	No	135, 140
Eastern eq. encephalitis	Unknown: 3-21 cases annually in the US	Little	No	No	182
Chikungunya virus	High: up to 75%	Yes	Yes	No	4, 145, 183-186
Mayaro virus	High: 5-60%	Yes	Yes	No	60, 89, 187-189
O'Nyong Nyong virus	High: 31-68%	Little	No	No	144, 145
Ross river virus	High: 8-65%	Yes	No	No	91, 135, 140
Sindbis virus	High: 5-27%	Yes	Yes	No	53, 82, 83, 146
Western eq. encephalitis	Unknown: <10 cases annually in US.	Little	No	No	40, 127, 147
Venezuelan eq. encephalitis	High: 30-50% during an epidemic. 23% outside epidemics. Up to 10% of dengue cases are thought to be VEEV in the Americas	Little	Yes	No	40, 60, 148, 190, 191

Symptoms*: FD = Febrile disease, AR = Arthralgia and/or Rash, HS = hemorrhagic syndrome, NS = Neurological syndrome. Circ.** = Circulation: R = Rural, U = Urban. CUD=Considered underdiagnosed. H2HT= Human-to-human transmission reported. Seroprev.= reported seroprevalence in local population

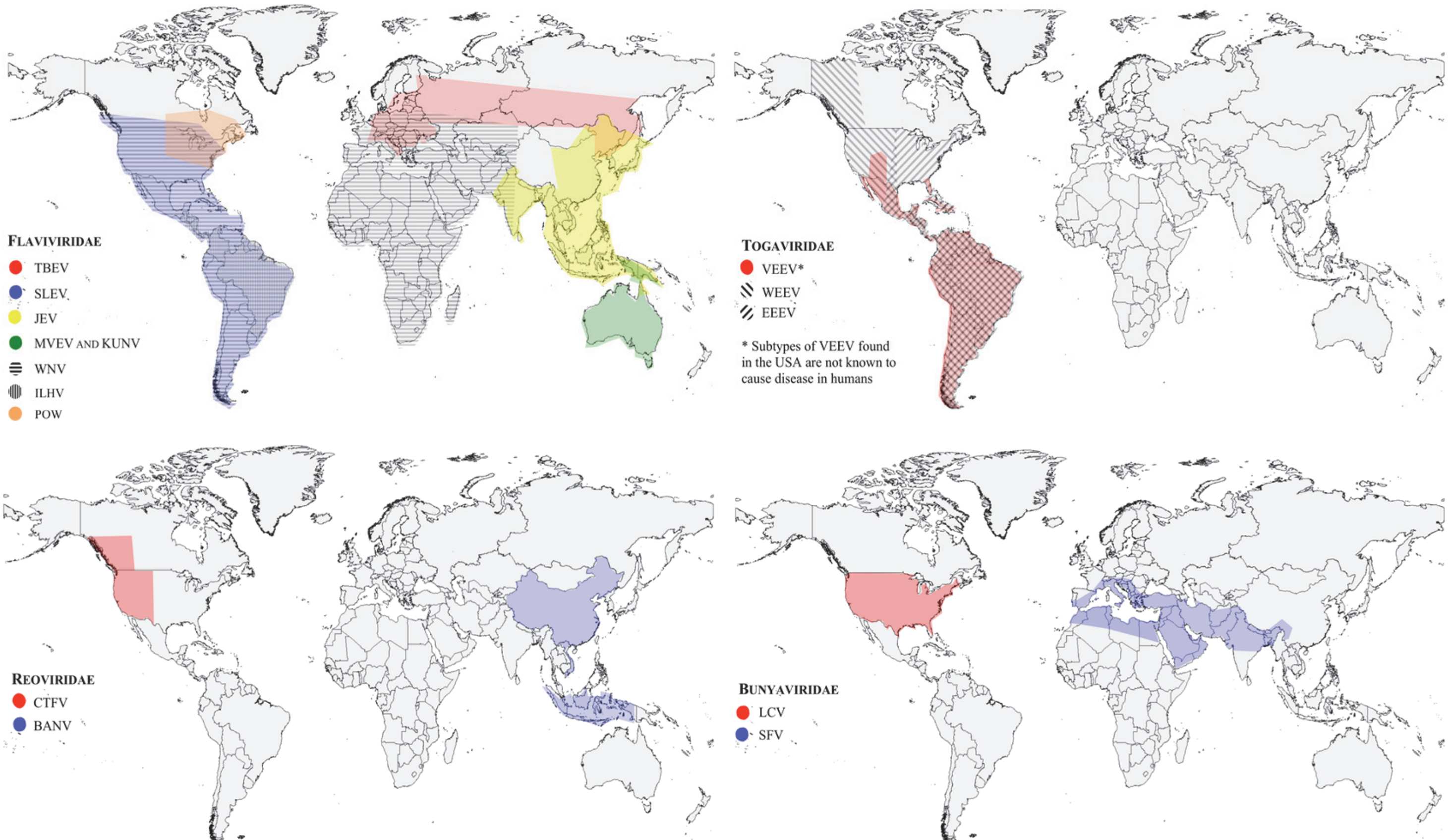
Table 3: Assessment of probability of arbovirus infections in travelers returning with illness, by travel destination and by main presenting syndrome.

Sub-region	Risk to travelers	FS	NS	HS	AR
North America (excl. Hawaii and Porto Rico)	More likely	WNV, SLEV, LCV	WNV, SLEV, LCV		
	Less likely	WEEV, EEEV, DENV, CTFV, ZIKV, CHIV	WEEV, EEEV, CTFV, CHIKV	DENV, CTFV	DENV, CTFV, WNV, CHIKV
	Unknown				
Central America and Caribbean	More likely	DENV, OROV, CHIKV, ZIKV		DENV	DENV, OROV
	Less likely	WNV, SLEV, WEEV, VEEV, EEEV	WNV, SLEV, OROV, WEEV, VEEV, EEEV		WNV, CHIKV, ZIKV
	Unknown	GROV, ILHV	ILHV		GROV
South America	More likely	DENV, YFV, OROV, CHIKV,, ZIKV		DENV, YFV	DENV, OROV, CHIKV, ZIKV
	Less likely	WNV, WEEV, VEEV, EEEV, SLEV	WNV, VEEV, WEEV, EEEV, SLEV, OROV		WNV
	Unknown	MAYV, GROV, ILHV	ILHV	MAYV	MAYV, GROV
Northern Africa	More likely	YFV, CHIKV, SFV		YFV	CHIKV
	Less likely	DENV, RVFV, WNV, CCHFV	WNV, RVFV, CCHFV	DENV, RVFV	DENV, CCHFV, WNV
	Unknown	BUNV, TAHV	BUNV, TAHV		BUNV, TAHV
Sub-Saharan Africa	More likely	DENV, YFV, CHIKV, ZIKV	WNV, RVFV	DENV, YFV	DENV, CHIKV, ZIKV
	Less likely	WNV, ONNV, SINV, CCHFV, RVFV	CHIKV	RVFV, CCHFV, CHIKV	SINV, ONNV, WNV
	Unknown	BWA, ILEV, TATV, TAHV, BUNV, NRIV	TAHV, ILEV, BUNV	ILEV, NRIV	TATV, TAHV, ILEV, BUNV, NRIV
Western and Central Asia	More likely	WNV, SFV, CCHFV		CCHFV	
	Less likely	RVFV, DENV, KFDV, AHFV, SINV	RVFV, KFDV, AHFV, CCHFV	RVFV, DENV, KFDV, AHFV	DENV, SINV
	Unknown	TAHV	TAHV		TAHV
Southeast, South and East Asia	More likely	DENV, CHIKV, ZIKV		DENV	DENV, CHIKV, ZIKV
	Less likely	JEV, WNV, KFDV, AHFV, SFV	JEV, WNV, KFDV, AHFV, CHIKV	KFDV, AHFV, CHIKV	WNV
	Unknown	BANV, CCHFV, TAHV	BANV, CCHFV, TAHV	CCHFV	BANV, TAHV
Oceania	More likely	RRV, BFV, MVE, ZIKV	MVE		RRV, BFV, ZIKV
	Less likely	DENV, JEV, KUNV	JEV, KUNV	RRV	DENV
	Unknown				
Northern Europe	More likely	SINV, TBEV	TBEV		SINV
	Less likely			TBEV	
	Unknown	TAHV	TAHV		TAHV
Southern Europe	More likely	TOSV, SFV, WNV	TOSV, WNV		
	Less likely	CHIKV, DENV, CCHFV	CCHFV	DENV, CCHFV	DENV, CHIKV, WNV
	Unknown	TAHV	TAHV		TAHV
Central Europe	More likely	TBEV	TBEV		
	Less likely			TBEV	
	Unknown	TAHV	TAHV		TAHV
Western Europe	More likely	TOSV, SFV (Southern France)	TOSV		
	Less likely	DENV, CHIKV, (Southern France)		DENV	DENV, CHIKV
	Unknown	TAHV	TAHV		TAHV
Eastern Europe (incl. Russia)	More likely	TBEV, WNV, SINV	WNV, TBEV		SINV
	Less likely	CCHFV	CCHFV	TBEV	CCHFV, WNV
	Unknown	TAHV	TAHV		TAHV

FD: Febrile disease; NS: neurological syndrome; HS: hemorrhagic syndrome; AR: Arthralgia and/or Rash. For full virus names see Table 1.

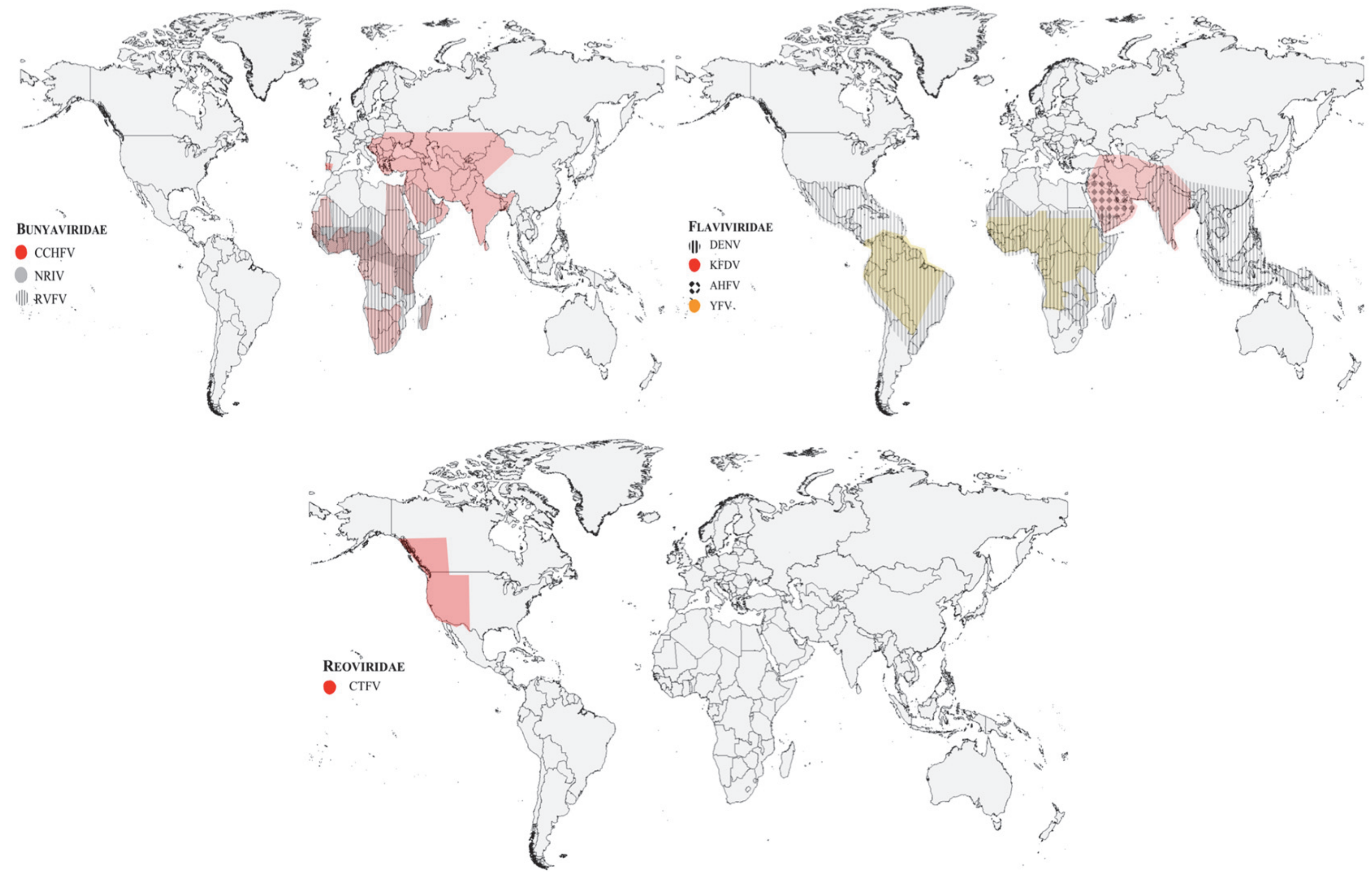
Map 1: Arboviruses that cause Neurological symptoms

General geographical overview of medically important arboviruses that cause neurological symptoms in humans based on Tables 1 and 2.



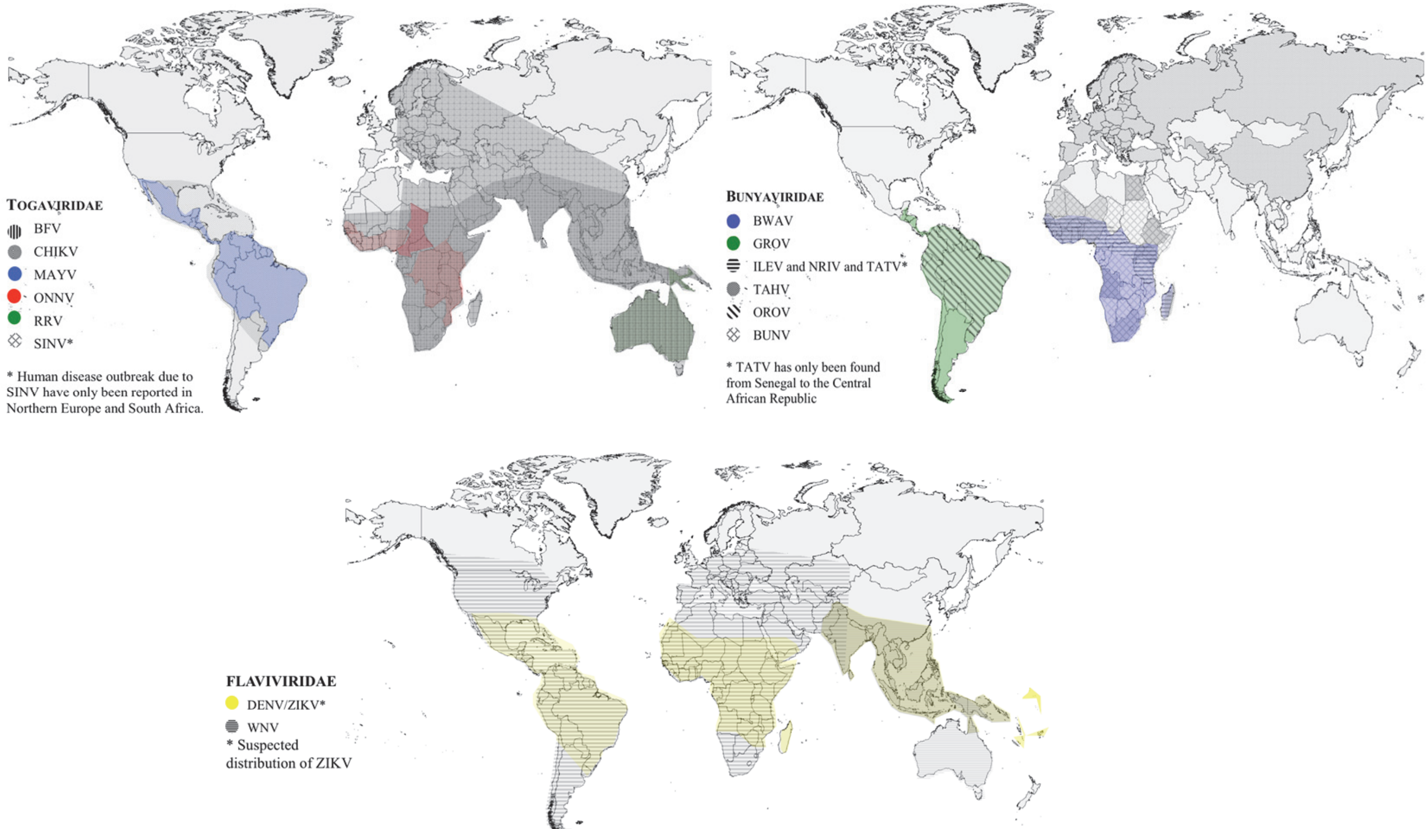
Map 2: Arboviruses that cause Hemorrhagic Symptoms

General geographical overview of medically important arboviruses that cause hemorrhagic symptoms in humans based on Tables 1 and 2.



Map 3: Arboviruses that cause Arthralgia and/or Rash

General geographical overview of medically important arboviruses that cause arthralgia and/or rash in humans based on Tables 1 and 2.



The problems with false positive diagnostic results, incomplete diagnostic range, availability of tests and costs of multiple tests illustrates that use of serology for diagnostics of travel-related viruses requires careful interpretation and the need for a detailed anamnesis to be provided with a diagnostic request. This includes travel history, vaccination history and contact with/ protection against specific vectors in combination with clinical symptoms and time of onset of symptoms (Table 3 and Map 1-3).[3]

Conclusion

Febrile disease in travelers has a long and complicated differential diagnosis including more common cosmopolitan infections as well as relatively unknown exotic diseases. Diagnostic tools can support clinicians and diagnostician in the construction of an adequate differential diagnosis. A few (commercially available) databases exist that can provide decision support tools for infectious diseases in general.[115, 116]

Many arbovirus families however, show a large amount of cross-reactivity in diagnostic tests. This, combined with a large overlap in clinical syndromes and geographical regions, complicates making a well-defined differential diagnosis. These complexities are not addressed in automatically generated differential diagnostic lists with a ranking based on unknown background information and assumptions.[116]

This review does not include all potential important travel-related diseases but focused specifically on all medically important arbovirus infections in travelers, taking the complexities of diagnostic test interpretation into account. Detailed maps, tables and supporting background information provided overviews on possible arbovirus infections per region. Thus, this review combined and structured the current knowledge per region, per clinical syndrome, per potential exposure risk, and per diagnostic family. In addition, this review identified pathogens for which the current knowledge in certain geographic regions is insufficient to exclude them in the differential diagnosis. With this, we hope to provide support to diagnosticians and clinicians when an arbovirus infection is considered in a returning traveler.

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Syndromic approach to arboviral diagnostics of global travelers as a basis for infectious disease surveillance

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Abstract

Background: Arboviruses have overlapping geographical distributions and can cause symptoms that coincide with more common infections. Therefore, arbovirus infections are often neglected by travel diagnostics. Here, we assessed the potential of syndrome-based approaches for diagnosis and surveillance of neglected arboviral diseases in returning travelers.

Method: To map the patients high at risk of missed clinical arboviral infections we compared the quantity of all arboviral diagnostic requests by physicians in the Netherlands, from 2009 through 2013, with a literature-based assessment of the travelers' likely exposure to an arbovirus.

Results: 2153 patients, with travel and clinical history were evaluated. The diagnostic assay for dengue virus (DENV) was the most commonly requested (86%). Of travelers returning from Southeast Asia with symptoms compatible with chikungunya virus (CHIKV), only 55% were tested. For travelers in Europe, arbovirus diagnostics were rarely requested. Over all, diagnostics for most arboviruses were requested only on severe clinical presentation.

Conclusion: Travel destination and syndrome were used inconsistently for triage of diagnostics, likely resulting in vast under-diagnosis of arboviral infections of public health significance. This study shows the need for more awareness among physicians and standardization of syndromic diagnostic algorithms.

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Introduction

Globalization has resulted in a steep increase in travel and trade.[1, 2] In recent decades it has contributed to the spread of diseases that traditionally emerged only regionally but now threaten populations across the globe, stressing the need for global health surveillance.[1, 2] Among these emerging threats, arboviruses form a unique group, with a large public health impact in endemic countries, a tendency to expand their geographical distribution through trade and travelers, and colonize previously unaffected areas. Due to their vector-borne and often zoonotic nature, they require targeted surveillance and control schemes.

This requirement is particularly relevant when evaluating symptoms of illness in travelers. Of all those returning from developing, tropical, or subtropical countries, 8% require medical care on return.[3] For those returning from Africa and Southeast Asia, fever is the most common reason for seeking medical care; for travelers returning from the Caribbean and South America, rash is the most common reason. Around 50% of the cases remain undiagnosed in clinics focused on travel medicine, and this percentage is likely higher in less specialized clinics.[3] The traveler's personal physician is therefore an important link in ongoing arbovirus surveillance in travelers and the gate-keepers of disease detection.

Correct diagnosis of arbovirus infections in travelers is challenging. Arboviruses have overlapping geographical distributions and cause symptoms that coincide with more common infections.[4] If general practitioners consider an arbovirus infection in their differential diagnosis, they commonly test for the best known arboviral disease, Dengue virus (DENV). Laboratory diagnostics for travelers are largely based on serologic testing, since viremia is short-lived and has often already dropped to undetectable levels when severe symptoms appear and diagnostics are performed.[5, 6] The use of serologic results for arbovirus diagnosis and surveillance requires careful evaluation due to cross-reactivity of antibodies to related viruses.[7] Also, several vaccines, notably for Yellow fever, Tick-borne encephalitis and Japanese encephalitis, can cause false-positive serological tests.[7]

For these reasons, arbovirus illness is under-diagnosed, as evidenced by studies of unexplained illness in returned travelers.[8-10] A potential solution would be the development of syndromic arboviral disease detection methods that cover the most common arboviruses and simultaneously provide surveillance information.[11] Here we aimed to assess the potential added value of syndrome-based approaches for diagnosis and surveillance of neglected arboviral diseases in returning Dutch travelers.

Method

To map the patients high at risk of missed clinical arboviral infections in returned Dutch travelers, we compared the quantity and quality of all arboviral diagnostic requests by Dutch physicians, from 2009 through 2013, with a previously extensive literature-based assessment of travelers' likely infection with an arbovirus.[4] The overlapping syndromes and geography, based on and updated from that review are depicted in figure 1.

Database construction

For retrospective patient analysis, a database was created by integrating data from the two arbovirus diagnostic reference centers in the Netherlands: Erasmus Medical Centre in Rotterdam and The National Institute for Public Health and the Environment in Bilthoven.

Previously, we described trends of DENV diagnostics in the Netherlands from 2000-2010.[12] The current study included almost all arbovirus diagnostic requests from Dutch physicians from 2009 through 2013 in the Netherlands. In the case of DENV not all data was included because 10% of the DENV diagnostics were performed outside the arbovirus reference centers and were not included in this dataset. For syndromic analysis, only entries were included where travel and clinical history were provided. To define the syndromes, entries in the database were reviewed by a consultant microbiologist, and infectious disease clinicians assigned them to syndrome categories (Table 1).

Patient test result classification

Due to the laboratory-specific variety in diagnostic methods used, we classified each patient's test results according to the validated methods and cut-offs for the pertinent laboratory. Results were classified as positive for a disease if the patient had (1) a positive PCR result with <40 cycles, (2) an IgM above an individual laboratory-determined cut-off, or [13] a minimum fourfold increase in IgG titers between two consecutive samples. For DENV patients, (4) a positive non-structural protein 1(NSI) antigen-capture test was among the criteria.[6]

Travel data

Travel data for Dutch travelers was based on the year 2011. They were extracted from a commercial database "ContinuVakantieOnderzoek" (CVO) created for trend analysis in the tourism industry. Its data are collected and converted into national numbers every three months by interviewing individuals in about 15,000 Dutch households on their travel destinations, activities, lodging, transport, and booking method.[14] Using data from 2011 provided a representative distribution of Dutch travel behavior from 2009-2011. Only slight country specific fluctuations were reported.[14]

Analysis of the likelihood of arboviral infections in travelers

The likelihood of infections by arboviruses other than DENV was based on a previously published article in which we developed syndromic diagnostic algorithms based on data from an exhaustive review of the literature addressing geographic distribution and prevalence of arboviruses by syndrome.[12] Optimal diagnostic algorithms using a combination of clinical syndromes and geographical distribution presented were updated and used as a basis for our current analysis (Figure 1). In short, criteria used to prioritize arboviruses for the diagnostic algorithm were: a) circulation in urbanized areas, due to the use of humans as reservoir hosts, or the presence of reservoir hosts colonizing urban areas, b) known endemic disease, c) tourist activity in the area, d) high rate of exposure in resident population, and e) recorded cases of infections in travelers[4] These diagnostic algorithms were used in the current article to identify gaps that may occur with a physician-indexed single-virus approach.

Statistical analysis

The analysis was performed in STATA.[15] Pearson's chi-square test was used to assess for equality of proportions. Multivariable logistic regression models (Table 2) reporting odds ratios were used with a 95% confidence level.[15] Heatmaps were generated using the additional R package "stats"[16] and based on pair-wise correlation between rows and columns.

Ethical statement
This research was conducted in accordance with the Dutch law on medical research (WMO), article 1. In compliance with Dutch Law and medical ethical guidelines, no personal identifiers were included and no informed consent was required for use of data in this study.

Results

General dataset
Over the five year study period 8126 patients were tested for arboviral diseases in the Netherlands. Of the patients, 44% presented to larger hospitals or specialized travel clinics. All other patients were seen at smaller hospitals or local clinics. Molecular tests comprised 1.3% of diagnostic tests performed. Larger hospitals and specialized travel/tropical clinics tested on average for 1.7 viruses per patient compared to 1.2 in smaller hospitals and local clinics. The patient male to female ratio was 1.04. Vaccination history was recorded on the diagnostic request for only 14 patients (<1%).
Of all patients, 2153 (26%) had information on travel history and clinical history and were thus included for further syndrome and travel-based analysis. Of these, 23% had provided a second serum sample needed for determination of a potential IgG titer increase. With a median of 7 days, the average number of days elapsed between onset of symptoms and first sampling was 17.5 (95%CI 14.0–20.3). This number is based on the 317 patients with clinical and travel history for whom this chronological information was recorded. Elapsed time did not differ between patients seen at specialized hospitals/clinics and those visiting smaller hospitals/clinics.

Comparison travel destination
We analyzed the travel data of Dutch travelers in 2011 to determine the range and importance of arbovirus tests needed to cover the differential diagnosis for travelers with illness after return from the various destinations. In 2011, approximately 84% of Dutch travelers traveling abroad stayed within Europe. Western Asia (predominantly Turkey) was the most popular non-Europea destination, with nearly one million Dutch vacations booked annually (Figure 2).[14] The most diagnostic requests (35%) by far, however, were for travelers returning from destinations in South and Southeast Asia, while only 3% of all travelers had this region as their destination.

Diagnostic requests and outcomes per region
The number of diagnostic requests by travel region and the proportion of positive test results (Figure 2) show that DENV testing was by far the most commonly requested (86%), yielding the highest absolute number of cases (Figure 2). When comparing the numbers of requests and proportions of positives by region of travel, substantial differences were observed: diagnostic requests for ill travelers returning from sub-Saharan Africa were frequent but not often positive, whereas ill travelers returning from popular arbovirus-endemic regions in Central and Western Asia were rarely tested. A low number of patients who had traveled within Europe were tested. DENV was tested (N=41) almost as often as tick-borne encephalitis virus (TBEV) (N=57), for which exposure is far more likely.

Table 1: Clinical manifestations classified per syndrome used for search in diagnostic database. Search was based on approximation of listed terms in multiple languages.

Syndromes						
Respiratory	**Enteric**	**Febrile**	**Neurological**	**Skin**	**Rheumatic**	**Hemorrhagic**
Throat ache	Diarrhea	Fever	Glasgow coma scale	Rash	Rheumatic	Secondary/ primary
Coughing	Vomiting	Pyrexia	Coma	Exanthema	pain	hemostasis
Wheezing	Dehydration	Febrile	Reduced responsiveness	Spots	Joint pain	Hematemesis
Hoarseness	Nausea	Temperature	Epileptic symptoms	Erythema	Arthralgia	Hemoptysis
Nasal / ocular discharge	Gastroenteritis	Malaise	Encephalitis	Maculopuritis	Arthritis	Melena
Bronchitis	Abdominal pain	Flu-like symptoms	Meningitis		Vasculitis	Hemorrhagic diatheses
Pneumonia			Myelitis			Thrombocytopenia
Rhinitis			Ataxia			Petechial
Hypoxia			Paresis			Ecchymosis
Dyspnea			Flaccid paralysis			Anemia
Apnea			Neurological symptoms			DIC (diffuse
Pleural effusion			Neurological dysfunction			intravascular
Chest congestion			Neurological disease			coagulation)
Earache			Polyradiculitis			Reduced clotting
Otalgia						Reduced platelet count
Sinusitis						
Epiglottitis						

4.1

Figure 1: Geographical distribution of medically important arboviruses that cause febrile disease in humans. All arboviruses cause febrile symptoms, but symptoms more specific to certain viruses are represented in three columns: 1) Arthralgia-Rash (AR); 2) Neurological symptoms (NS), and 3) Hemorrhagic symptoms (HS). Virus name is written in the color of its corresponding (and potentially cross-reactive) family. Arboviruses not known to cause more than febrile symptoms are preceded with a §-sign. Arboviruses more likely to be diagnosed in travelers are followed by *. Geographical regions based on UN definitions of world regions. EU, Sub-Saharan Africa and South & Southeast Asia regions are grouped in these representations for visual clarity but are subdivided according to UN definitions for analysis as can been seen in figures 3-6.

North America
AR: WNV*, DENV^, ZIKV, CHIKV
NS: CEV/LCV*, WNV*, POWV, SLEV, EEEV, WEEV, CTFV
HS: DENV^

Caribbean and Central America
AR: DENV^*, WNV, ZIKV*, OROV*, CHIKV*
NS: OROV*, WEEV, EEEV, VEEV, ILHV, WNV, SLEV
HS: DENV^*

South America
AR: DENV^*, ZIKV*, WNV, CHIKV*, MAYV*, OROV*
NS: OROV*, WEEV, EEEV, VEEV, SLEV, WNV, ILHV, ROCV
HS: DENV^*, YFV

Europe
AR: WNV*, DENV^, TAHV, SINV*, CHIKV
NS: TBEV*, WNV*, LIV, TOSV*, BATV, TAHV
HS: DENV^, CCHFV, §SFV*

North Africa
AR: DENV^*, WNV*, CHIKV*, SINV, TAHV, BUNV
NS: TOSV*, RVFV*, TAHV, §SFV*
HS: RVFV*, CCHFV*, YFV*, DENV^*

West and Central Asia
AR: DENV^*, WNV*, TAHV, SINV, §SFV*
NS: CHIKV*, WNV*, TBEV, BANV, TAHV, RVFV*
HS: RVFV*, CCHFV, DENV^*, OHFV, AHFV

Sub-Saharan Africa
AR: DENV^*, WNV*, YFV*, ZIKV, CHIKV*, SINV, ONNV, BWA, TAHV, ILEV, TATV, NRIV
NS: WNV*, RVFV*, BUNV, TAHV, BWA, BUNV, ILEV
HS: DENV^*, RVFV*, NRIV, ILEV, CCHFV
§WSBV

East Asia
AR: DENV^*, WNV, CHIKV*, TAHV
NS: JEV*, TBEV, WNV, BANV, TAHV
HS: DENV^*, OHFV, SFTSV, CCHFV

South and Southeast Asia
AR: DENV^*, WNV*, ZIKV*, TBEV, CHIKV*, TAHV
NS: JEV*, WNV*, TBEV, BANV, TAHV
HS: DENV^*, KFDV, SFTSV, CCHFV

Oceania
AR: RRV*, BFV*, CHIKV, SINV, WNV, DENV^, ZIKV
NS: MEV*, JEV, WNV
HS: DENV^

Flaviviridae
Togaviridae
Bunyaviridae
Reoviridae

AKHV = Alkhurma hemorrhagic fever virus; BANV = Banna virus; BFV = Barmah Forest virus; BWAV = Bwamba virus; BUNV = Bunyamwera virus; CEV = California encephalitis virus; CHIKV = Chikungunya virus; CTFV = Colorado tick fever virus; CCHFV = Crimean-Congo hemorrhagic fever; DENV = Dengue virus; EEEV = Eastern equine encephalitis virus; GROV = Guaroa virus; ILEV = Ilesha virus; ILHV = Ilheus virus; JEV = Japanese encephalitis virus; KFDV = Kyasanur Forest disease virus; LCV = La cross virus; LIV = Louping Ill virus; MAYV = Mayaro virus; MURV = Murray Valley virus; NRIV = Ngari virus; OHFV = Omsk hemorrhagic fever virus; ONNV = O'Nyong Nyong virus; OROV = Oropouche virus; RVFV = Rift Valley fever virus; ROCV = Rocio virus; RRV = Ross river virus; SFV = Sandfly fever (Naples / Sicilian / other); SFTS V = Severe Fever with Thrombocytopenia Syndrome Virus; SINV = Sindbis virus; SLEV = St. Louis encephalitis virus; TAHV = Tahyna virus; TATV = Tataguine virus; TBEV = Tick-borne encephalitis virus; TOSV = Toscana virus; VEEV = Venezuelan equine encephalitis virus; WEEV = Western equine encephalitis virus; WNV = West Nile virus; YFV = Yellow fever virus; ZIKV = Zika virus.

Of note, two of these European travelers tested DENV-positive. One was a tourist returning from Croatia, who tested DENV-IgM-positive and borderline NS1-positive. The other tourist had taken a five-day trip to Southern France and was DENV IgM- and NS1-positive 10 days after return. However, 14 days previous to onset of symptoms, this traveler had been in Thailand before traveling on to France. Another virus considered endemic to Europe is Sindbis virus (SINV), for which diagnostics are not readily available in the Netherlands. Nor are they available for oropouche virus (OROV), endemic to South America.

Syndromes reported

To assess the potential use of diagnostic requests for syndrome surveillance by region, we analyzed the symptoms recorded for each patient returning from a particular travel destination. Nearly all patients (86%) reported fever, followed by arthralgia/arthritis (22%) and enteric symptoms (14%). Information divided per travel region showed regional variation in symptoms recorded (Figure 3). For all regions, fever was the most reported symptom. Proportionally, neurological symptoms were more often reported for travelers returning from a European destination than for travelers from other regions. Arthralgia-arthritis was recorded more frequently for travelers returning from Oceania, with rash being most recorded for Southern Africa compared to other regions.

Comparing diagnostic requests to diagnostic algorithms

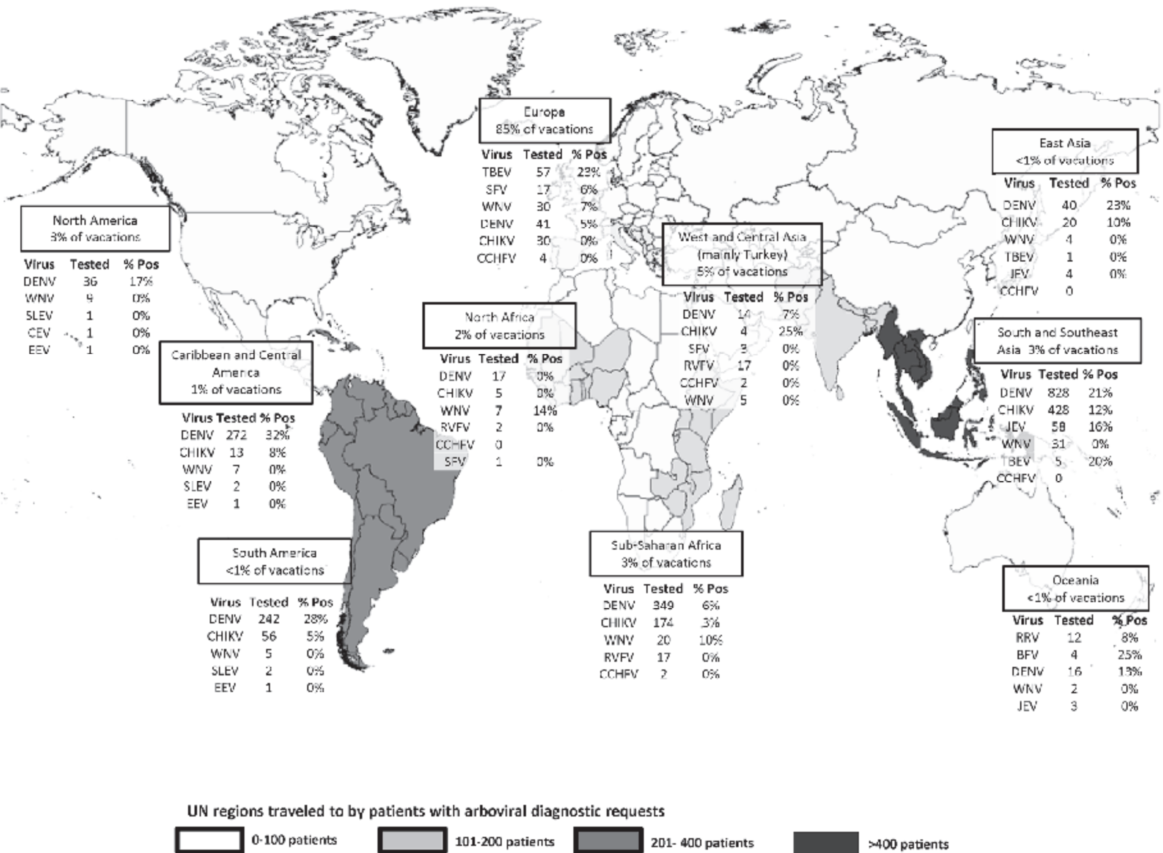
Three heatmaps were created to visualize per continent (Africa, Asia and the Americas) the correlation between the physicians' diagnostic requests and the literature-based syndromic algorithms (Figure 1). In the heatmaps, diagnostic requests are grouped based on the clinically important arboviral diseases per region within each continent (Figure 4-6). For most regions, Dutch physicians requested DENV diagnostics for 100% of the travelers who had recorded symptoms corresponding to DENV infection (fever, rash and joint pain). For some regions, a lower percentage of such patients was tested, i.e. Northern Africa (67%) (Figure 4), Western Asia (57%) (Figure 5) and Central America (38%) (Figure 6).

In all regions, CHIKV testing was less frequently requested than DENV testing, even though the infections overlap in geographical distribution and range of symptoms to a great extent. On average, 45% of patients with febrile symptoms, rash and/or arthralgia after travel to CHIKV-risk areas in Asia were not tested for CHIKV. Patients with symptoms suggesting West Nile Virus (WNV), Japanese encephalitis (JEV), Rift Valley fever virus (RVFV) and TBEV were tested infrequently (0 to 25%) and only in association with neurological symptoms. Diagnostics on all other viruses presented in figure 4-6 were minimally requested.

Predictive factors for positive tests

We analyzed the association between symptoms recorded and test outcomes for DENV and CHIKV requests in Dutch travelers (Table 2). Patients with rash, hemorrhagic symptoms and fever had an increased odds of testing positive for DENV, but respiratory symptoms decreased the odds of being DENV-positive (OR 0·5). Positive test outcomes for CHIKV were associated with arthralgia combined with rash. Both DENV and CHIKV were positively associated with travel history to Southeast Asia.

Figure 2: Geographical depiction of the number of diagnostic tests requested after travel to each region (see gray shading and tables) from 2009 to 2013. Boxes show number and percentage of all vacations booked from the Netherlands to each region in 2011.



Discussion

Here we assessed the extent of missed arboviral infections in travelers by a retrospective database analysis of all arboviral diagnostic requests in the Netherlands, from 2009-2013, in comparison with a literature-based assessment of arbovirus exposure while traveling (Figure 1). We found clear evidence for patient groups high at risk of being under-diagnosed for arboviral disease when evaluated by syndrome and by region.

While DENV diagnostics are routinely requested, other relevant arboviruses are neglected, in particular CHIKV. Arthralgia, for example, is not only associated with DENV infections but also with many arboviruses, including CHIKV, as we found when calculating odds ratios within the current Dutch data.[4] Nevertheless, less than 55% of patients with symptoms compatible with CHIKV infection were tested (Figure 4a-c).
Interestingly, hemorrhagic symptoms and rash have a much higher odds ratio than arthralgia-arthritis for diagnosing DENV. Although arthralgia is an important symptom in dengue patients, rash and fever are often more pronounced.[17] In the case CHIKV arthralgia-arthritis is more pronounced and is known to have a higher predictive value for distinguishing CHIKV from DENV in endemic settings.[17, 18] Additionally, CHIKV is less well known by physicians in non-endemic countries so might be only considered if DENV diagnostics are negative.

The analysis of diagnostic requests by region showed a bias toward the more well-known arboviral risk areas such as Southeast Asia (Figure 2 and 3). For travelers within Europe, arbovirus diagnostics are rarely requested, despite high incidence rates of TBEV reported across Europe and continuing circulation of WNV in parts of Europe popular with Dutch tourists.[8, 19] This is a general trend also seen in previous reports on travel associated infection presenting in Europe.[20] Housing type and location during travel is an import risk factor for exposure to specific vectors,[21, 22] and outdoor camping is popular among travelers in Europe.[14] The number of CHIKV and DENV requests within Europe was almost equivalent to the number of TBEV and WNV test requests, while only a small number of CHIKV and DENV have been reported.[23-25] The low number of TBEV and WNV requests may reflect a lack of physician awareness of European arboviruses and their risk to travelers; it may also reflect financial restrictions or limited time.[19]

Our analysis showed that physicians were more likely to extend the diagnostic panel for patients with more severe or very specific symptoms. For instance, diagnostics for WNV and Western equine encephalitis virus (WEEV) were usually requested only for patients with neurological complaints, even though fever is the most common clinical presentation in >90% of WNV and WEEV patients.[26] Similarly, RVFV diagnostic requests were limited to patients with hemorrhagic symptoms (HS) and neurological symptoms (NS), although these severe symptoms occur in less than 1% of cases, and most patients present only with febrile symptoms (Figure 4a-c).[27]

Figure 3: Percentage of patients (left axis) with arbovirus diagnostic requests presenting with symptoms by travel destination (horizontal axis). The number of patients per group is shown in parentheses on the horizontal axis (based on 2153 patients with both travel and clinical history).

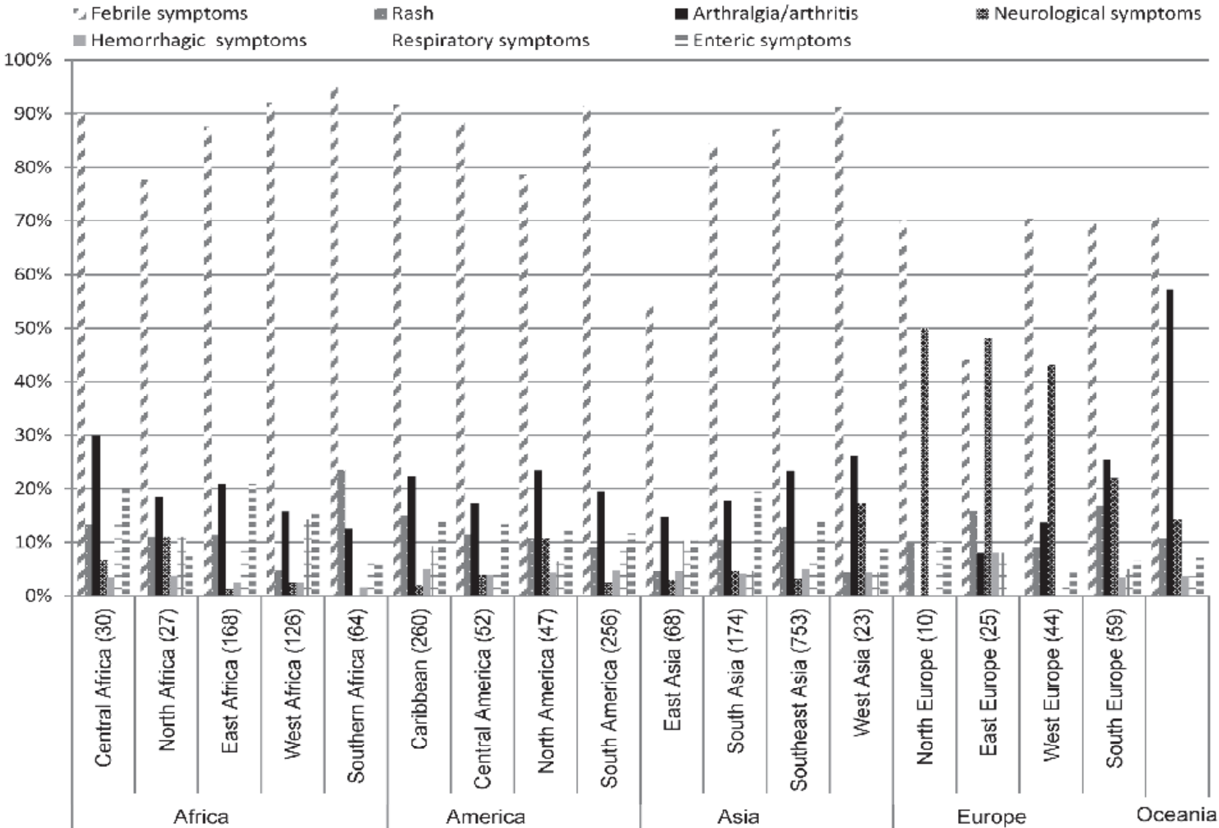


Figure 4-6: Heatmaps showing percentage of patients with a travel history to (4) Africa, (5) Asia or (6) Americas, divided by region (right axis) and recorded symptoms (left axis), who were tested for each arbovirus (horizontal axes) posing a risk on that continent (see Figure 1). The number of patients in each region-symptom combination follows each region in parentheses, far right. Groups in which a 100% of patients with a specific region-symptom combination were tested are depicted as black, with a sliding scale to white for groups in which 0% of patients were tested. Region-symptom combinations that are atypical for a certain arbovirus are depicted as diagonal lines.

Figure 4: Africa

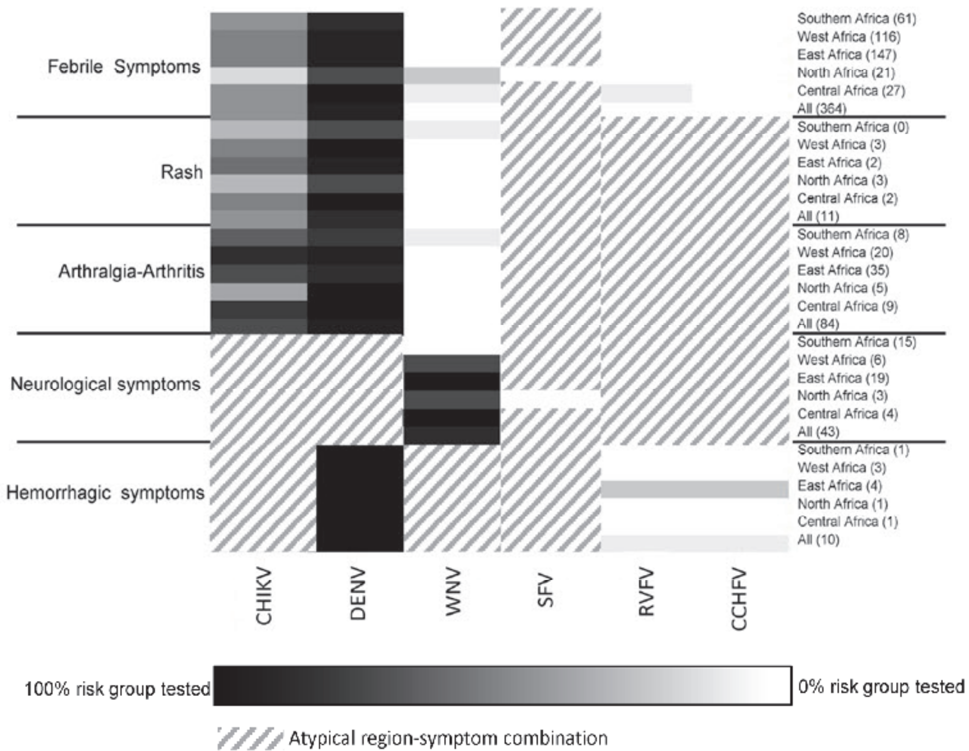


Figure 5: Asia

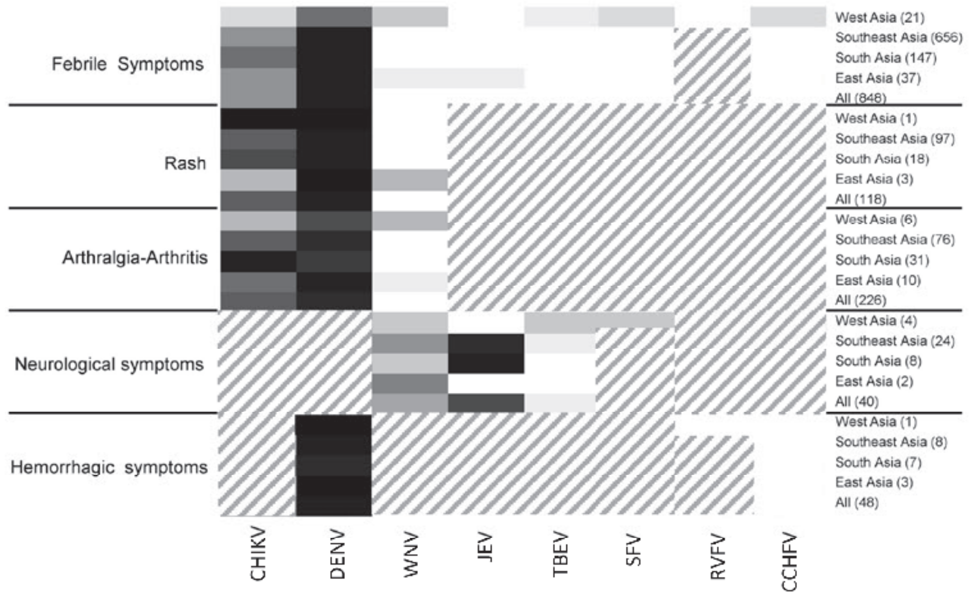


Figure 6: Americas

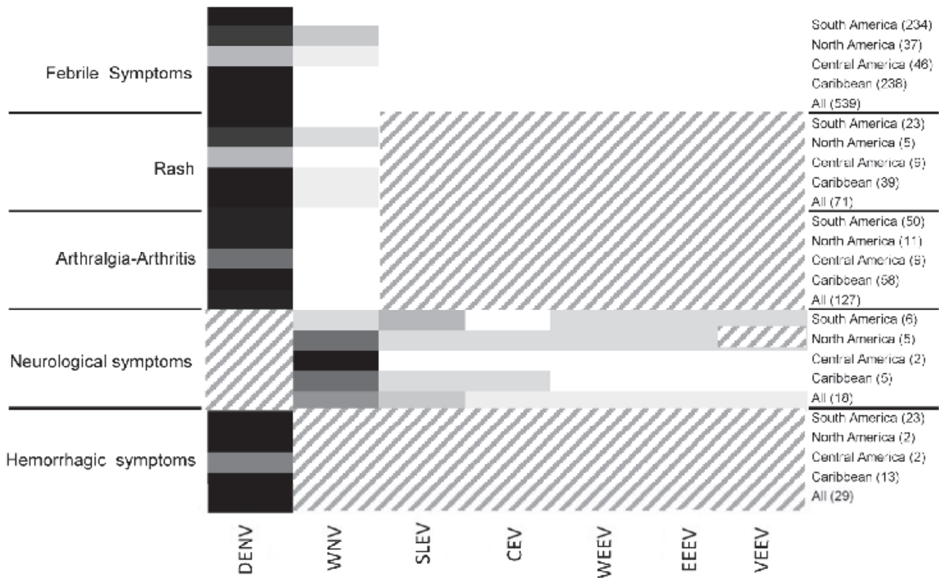


Table 2: Adjusted odds ratios of statistically significant predictive syndromes for a positive test outcome. The test is stated in column 1, with corresponding variables in column 2. Variables were adjusted for age, sex, travel region, and diagnostic laboratory**.**

Dependent variable	Independent Variable	Adjusted Odds ratio	95% CI	P-value
DENV-positive versus negative test outcome (based on 1843 patients with DENV-diagnostic tests performed)	Febrile symptoms	2.0	1.2 - 3.0	*<0.01*
	Rash	1.9	1.3 - 2.5	*<0.01*
	Arthralgia-arthritis	0.5	0.3 - 0.8	*<0.01*
	Hemorrhagic symptoms	2.8	1.8 - 4.5	*<0.01*
	Neurological symptoms	0.7	0.2 - 1.9	0.4
	Respiratory symptoms	0.5	0.3 - 0.8	*<0.01*
	Enteric symptoms	0.8	0.5 - 1.1	0.1
CHIKV-positive versus negative test outcome (based on 736 patients with CHIKV-diagnostic tests performed)	Febrile symptoms	1.5	0.7 - 3.2	0.3
	Rash	4.0	2.2 - 7.1	*<0.01*
	Arthralgia-arthritis	2.9	1.7 - 5.2	*<0.01*
	Hemorrhagic symptoms	0.4	0.1 - 3.4	0.4
	Neurological symptoms	0.7	0.1 - 6.2	0.7
	Respiratory symptoms	0.3	0.1 - 1.1	0.1
	Enteric symptoms	0.4	0.1 - 1.1	0.1

This bias toward severe symptoms was likewise reflected by the finding that patients referred to large hospitals and travel clinics were more extensively evaluated than those visiting small hospitals and local clinics. Reasons for this difference were not assessed in our study but are likely related to the fact that 1) general practitioners often omit arbovirus diagnostics, due in part to budgetary constraints; 2) they may lack knowledge on arboviral disease, and 3) may believe that an arbovirus diagnosis is unlikely to influence their treatment decisions, particularly if symptoms are mild.
However, even mild arbovirus infections can eventually cause severe or chronic symptoms like arthralgia and, in any case, they pose a potential risk to health workers. Lack of proper diagnosis may lead to unnecessary complications or extensive later testing of patients. A possible solution to this problem is diagnostic centers providing syndromic and region based diagnostic packages for travelers as presented by the algorithms here.[4] These can be continuously updated in collaboration with specialized physicians and Public Health professionals. This will relieve the general physicians from keeping up to date on such a complex and continuously changing area. At the same time physicians are provided with a complete diagnostic selection and data are more suitable for use in surveillance.

Our results show a large variation in the timing of first diagnostic sampling. In our study, 50% of travelers contacted a healthcare provider during the first week of illness. This means that 50% did not, and viremic patients may introduce viruses into a region, when appropriate vectors are available, [24, 28] or pose a risk for nosocomial infection.[29, 30]
Only 1.3% of all diagnostic tests performed were molecular, while 50% of patients fell within the range advised for molecular testing. The timeframe for molecular and serological diagnostics overlap to a great extent. Within the first days of illness, however, serology has a low sensitivity.[6] A number of the DENV cases may have been secondary, tertiary or quaternary infections. This reduces the sensitivity of serological detection by IgM in non-primary infections significantly.[6] Many patients are therefore probably missed due to lack of molecular testing within this timeframe.
To use diagnostic data for syndromic surveillance, a two-tiered approach could be employed. First, samples collected after three days of illness onset would provide syndromic information by multiplex serologic testing. Second, if testing showed increased circulation of a target virus, confirmation and genomic surveillance would follow in patients suspected to harbor that virus sampled within seven days of illness.

There are a number of limitations to this study. Nearly all patients tested for arboviral diseases in the five-year-period in the Netherlands were included. This group, however, only consists of patients that seek medical attention after travel and that are suspected of an arboviral infection by a clinician. Asymptomatic patients and patients where clinicians did not consider an arboviral disease are missed.
Almost all patients lack vaccination history. Patients with recent yellow fever vaccinations could cause positive false positive serological tests.[6] Lack in reporting vaccination history and the resulting possible flavivirus cross-reactivity due to vaccination are known problems when using flavivirus serological diagnostic data.[9] Both diagnostic centers had extensively validated tests internally with yellow fever vaccines and changed diagnostic cut-offs provided by manufacturer to compensate if possible. However, false positive tests due to vaccination cannot be excluded.

Infectious disease diagnostics and surveillance of travelers is primarily focused on those cases or diagnostic outcomes selected and reported by physicians.[31-34] Although this approach provides essential information, many patients remain undiagnosed, and re-evaluation of the selected pathogens has been advised.[10, 32, 35]
However, much knowledge on probable arbovirus exposure of travelers is based on information originating from the destination country, which may have limited surveillance and diagnostic capabilities. In some of these countries, large-scale surveillance projects using a more syndromic approach to infectious diseases have shown extensive under-diagnosis and under-recognition of the importance of many arbovirus diseases as a cause of common syndromes.[36, 37]
This underlines the need, in the Netherlands and other affluent countries, for more systematic syndrome-based diagnosis and surveillance in travelers to these regions. It demonstrates the added value of using routine travel information to support national and international surveillance programs. For such surveillance, capturing only a fraction of all cases may still provide reliable information on disease trends and possibly local outbreaks, provided the selection is systematic.[9] It is also important in terms of preparedness for emerging infectious diseases.

Conclusion

A physician's diagnostic requests for returned travelers can play a key role in infectious disease surveillance. However, while travel destination and syndrome could be used for triage and diagnostics, such use is inconsistent. We found clear evidence of patient groups at risk of under-diagnosis of arboviral disease when evaluated by syndrome and by region.
Based on a comparison between all arboviral diagnostic requests by physicians in the Netherlands between 2009 and 2013 with a literature-based assessment of the likely exposure of the patients to an arbovirus, we showed that while dengue virus diagnostics are routinely requested, other relevant arboviruses such as chikungunya virus are neglected, even if travelers present with relevant symptoms and return from countries where the viruses are endemic. We also showed that for travelers to European destinations, arbovirus diagnostics were rarely requested and that for almost all arboviruses and travel destinations, diagnostics were requested only when patients presented with severe symptoms.
Whether the low number of requests and overemphasis of physicians on patients presenting with severe symptoms reflects a lack of physician awareness of arboviruses and their risk to travelers, financial restrictions or limited time, it points at possible gaps in preparedness. Our paper shows that in order to limit the amount of missed clinical arboviral infections, and to increase the level of awareness of arboviral infections of public health significance, physicians should rely on diagnostics and surveillance with a syndromic approach and matching laboratory methods.

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4.2

4.2

Ross River virus disease in two Dutch travellers returning from Australia, February to April 2015

Reusken C., Cleton N., Medonca Melo M., Visser C., GeurtsvanKessel C., Bloembergen P., Koopmans M., Schmidt-Chanasit J., and Van Genderen P.

Abstract

We report two cases of Ross River virus (RRV) infection in Dutch travellers who visited Australia during February to April 2015. These cases coincided with the largest recorded outbreak of RRV disease in Australia since 1996. This report serves to create awareness among physicians to consider travel-related RRV disease in differential diagnosis of patients with fever, arthralgia and/or rash returning from the South Pacific area, and to promote awareness among professionals advising travellers to this region.

Euro Surveill. 2015 Aug 6;20(31). pii: 21200

Case presentation

Case 1

A woman in her early 50s with a history of polymyalgia rheumatica visited the outpatient department of a hospital in Rotterdam because of persistent joint pains after travel to Australia. She had stayed in Australia from 30 January until 5 March, where she mainly stayed in the surroundings of Perth. From 7 February, she stayed in Cairns for six days. She recalled having had multiple mosquito bites during her stay in Cairns. Seven days after her return to Perth (on 20 February), she developed fever, fatigue, frontal headache, muscle aches and arthralgia of her hands, wrists, feet and ankles. In addition, she noticed an itchy papular rash on her face, neck and trunk. She was treated with prednisone by a local general practitioner for a presumed recurrence of her polymyalgia, pending the results of serological investigations. Serology for RRV was IgM positive, therefore treatment with prednisone was discontinued.

Two months after returning to the Netherlands, she still experienced debilitating arthralgia and an unsteady gait, frequently necessitating the use of a walking aid. In addition, she reported a subfebrile temperature and sweating. On physical examination, no abnormalities were seen. She had a normal body temperature of 36.9 °C and her joints did not show any sign of arthritis. Laboratory investigation revealed an elevated erythrocyte sedimentation rate (ESR) of 32 mm/hr, a normal leukocyte count of 6.6 x 109/L, no abnormalities in the differential morphology of the leukocytes and a C-reactive protein (CRP) level of 6 mg/L (norm: <10 mg/L). Serological testing for RRV on convalescent serum (taken 7 April) showed the presence of IgM and seroconversion for IgG antibodies specific for RRV (Table). RRV aetiology was further confirmed by comparative indirect immunofluorescence assay (IIFA) for RRV, Barmah Forest virus (BFV), chikungunya virus (CHIKV) and Sindbis virus (SINV), and virus neutralization (Table). BFV, CHIKV and SINV are alphaviruses causing symptoms comparable to those caused by RRV, which are endemic to the region.

Case 2

A woman in her late 60s visited her general practitioner on 11 May 2015 with complaints of fatigue, myalgia, arthralgia and a maculopapular rash but no fever. The patient had visited Australia from 29 March to 9 May 2015, where she stayed in New South Wales (in Sydney, Armidale and a mangrove forest near Coff Harbour). She recalled having been bitten by mosquitoes during a trip on 14 April. The first symptoms of wrist pains appeared around 21 April, followed by a rash a few days later. The patient visited a local physician on 27 April and treatment with meloxicam was initiated. Laboratory investigation revealed a normal erythrocyte sedimentation rate of 5 mm/hr. Diagnostics for RRV, BFV, Epstein–Barr virus, B19 parvovirus and connective tissue disease were negative.

Upon the patient’s return to the Netherlands, the rash reappeared (Figure) and the joint pains in her hands and knees increased. Treatment with naproxen was started. Serology for Borrelia burgdorferi showed IgM but no IgG; however, this diagnosis remained inconclusive as it was not confirmed by analysis of a second serum sample. Serological testing for RRV and BFV on a convalescent serum taken on 20 May showed the presence of IgM and IgG antibodies specific for RRV (Table). A second serum sample taken on 24 June showed decreasing IgM and increasing IgG titres. RRV aetiology was further confirmed by comparative IIFA for RRV, BFV, CHIKV and SINV, and by virus neutralisation (Table).

Table: Differential diagnostics for two Dutch travellers returning from Australia with Ross River virus disease, February to April 2015

Antibody tested	Test results	
	Case 1 Sample taken 46 days after symptom onset	Case 2 Sample taken 30 days after symptom onset
Anti-RRV-IgG[a]	2.23	3.64
Anti-RRV-IgM[a]	4.81	6.33
Anti-RRV-IgG[b]	1:10,240	1:2,560
Anti-RRV-IgM[b]	1:640	1:5,120
RRV NAb[c]	1:40	1:40
Anti-BFV-IgG/IgM[a,b]	neg	neg
Anti-CHIKV-IgG[b]	1:160	1:1,280
Anti-CHIKV-IgM[b]	1:320	neg
CHIKV NAb[c]	neg	neg
Anti-SINV-IgG/IgM[b]	neg	neg

BFV: Barmah Forest virus; CHIKV: chikungunya virus; NAb: neutralising antibodies; neg: negative; RRV: Ross River virus; SINV: Sindbis virus.

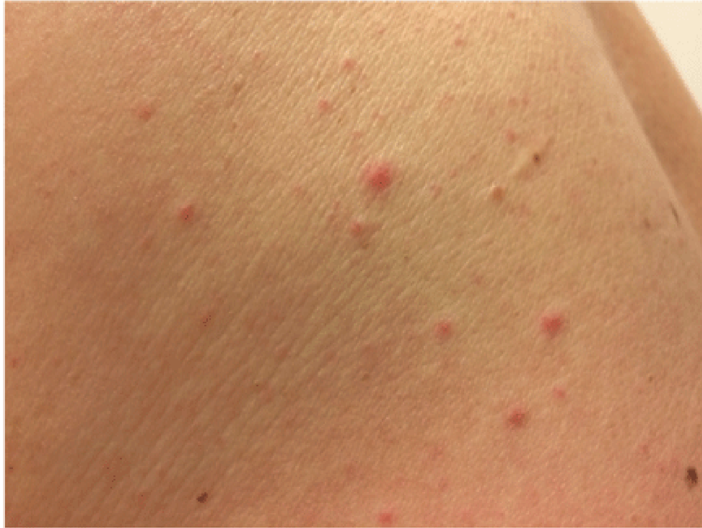
[a] Enzyme-linked immunosorbent assay (PanBio ELISA) values <1.0 were considered negative.

[b] Indirect immunofluorescence assay titres <1:20 for serum were considered negative [9].

[c] Virus neutralisation test titres <1:20 for serum were considered negative [9].

4.2

Figure: Rash in a traveller (Case 2) returning from Australia with Ross River virus disease, 19 May 2015, 29 days post symptom onset



Background

RRV, an alphavirus transmitted by mosquitoes, is endemic in Australia and Papua New Guinea, with occasional epidemics in island countries in the Asia-Pacific region. The virus is maintained in an enzootic cycle between mosquitoes and marsupials, such as kangaroos and wallabies, as primary reservoirs. Rodents, rabbits, fruit bats, possums, horses, cats and dogs have been implicated as well [1-3]. Human-to-human transmission has been described in epidemic situations, and viraemic travellers from Australia have been linked to epidemics in the Cook Islands, Fiji, New Caledonia and Samoa [3]. Unnoticed circulation of RRV has been described in French Polynesia [4]. Recent evidence supports RRV transmission through blood donation [5]. Mosquitoes belonging to the genera Aedes and Culex are considered the main vector species and vertical transmission has been described as a way for the virus to persist during adverse conditions in desiccation-resistant eggs [6].

RRV is endemic in tropical and subtropical Australia (Northern Territory and Queensland) with year-round notification of human cases, while in temperate Australia (New South Wales and Victoria) human cases occur seasonally and in epidemics [3,6]. According to the Australian Department of Health, by 23 June 2015, a total of 7,552 RRV disease cases had been reported this year, which is the largest number of annual reported cases since 1996 [7]. Most cases up to 23 June were reported in Queensland (n = 5,075) and New-South Wales (n = 1,292) and peak incidences were in February to April.

The incubation period for RRV disease (also called epidemic polyarthritis) is typically 7–9 days, ranging from 3 to 21 days [1]. In 55–75% of infections, the individuals are asymptomatic. Symptomatic disease typically includes arthralgia, myalgia and fatigue.

Low-grade fever (37.5–38.5 °C) and maculopapular rash on the torso and limbs (sometimes palms, soles and face) occur in 50–60% of clinical cases [1]. Joint pain, stiffness and swelling are usually symmetrical, affecting wrists, hands, fingers, ankles and knees. Additional manifestations may include headache, diarrhoea and nausea. Symptoms most often resolve within 3–6 months; permanent sequelae have not been described.

Treatment of symptomatic cases is supportive. Analgesics and nonsteroidal anti-inflammatory drugs may be helpful in the treatment of arthritis and arthralgia. No vaccine is available [1,3].

Laboratory findings are non-specific. Leukocyte counts and CRP levels are usually normal, ESR elevated. Diagnostics are most often based on serology as the viraemic stage is very short (typically fewer than seven days post symptom onset for alphaviruses) and molecular diagnostics are not considered useful on samples taken more than a week after symptom onset. Serology is complicated by putative cross-reactivity with other alphaviruses, especially CHIKV, which belongs to the same serogroup [1,3,8].

Discussion

Diagnosis of RRV disease in travellers returning to Europe is very rare [8-11]. A history of mosquito bites and stay in RRV-affected areas are epidemiological parameters supportive of a confirmative diagnosis based on RRV IgM and IgG responses. Based on these criteria, only three cases of RRV disease have been confirmed between 1 January 2009 and 30 June 2015 in the Netherlands, including the two cases in 2015 reported here, who had additional confirmation by gold-standard serology (virus neutralisation). In this period, a total of 56 diagnostic requests for RRV were submitted to the Dutch national arbovirus reference centre in Rotterdam. Of these, 20 requests indicated the travel destination as Australia and/or Asia-Pacific; for 30 requests, the travel destination was unknown. Although local circulation of RRV is unknown, travel to Indonesia, Thailand, Malaysia and the Philippines triggered diagnostic requests as well. Febrile disease and/or arthralgia were the most common symptoms (in 21 of 35 diagnostic requests with clinical data), leading to RRV disease being considered.

The cases presented here highlight the importance of considering RRV in differential diagnosis for travellers presenting with acute arthritis returning from Australia and the Asia-Pacific region. The two cases we describe were related to travel to risk areas in Australia in February/March and March–May 2015. Other arboviruses circulating in Australia and causing polyarthritis that should be included in differential diagnosis are the alphaviruses BFV and SNV, and the flaviviruses West Nile virus (Kunjin virus, KUNV) and Kokobera virus (KOKV). Depending on other travel destinations in the Asia-Pacific region, chikungunya virus, dengue virus and Zika virus should be considered as well [3,12]. The annual incidence of RRV disease in Australia ranges from 2,000 to 8,000 cases; for BFV disease, from 500 to 2800. Human cases of KUNV or KOKV disease are rare, while the occurrence of human infections with the Oceania lineage of SINV is under debate [3,12].

Although RRV-viraemic travellers have been linked to the spread and epidemics with RRV in the Asia-Pacific region, it is highly unlikely that return of viraemic travellers to Europe will result in autochthonous transmission. As the duration of viraemia is short, the likelihood that a traveller will be viraemic on their return is small. More importantly, the three main vectors for RRV transmission based on field isolations and competence studies are either strictly confined to Australia (Ae. vigilax and Ae. camptorhynchus, both invasive but not established in New Zealand) or the Asia-Pacific region (C. annulirostri) (1).

This report underlines the need for awareness of RRV-related risks among physicians, professionals advising travellers and travellers themselves. Australia is a popular travel destination for Europeans, especially for German, British and French tourists [13]. The number of leisure travellers from the Netherlands to Australia and/or New Zealand has been stable during 2002 to 2011, averaging to 52,000 travellers per year [14]. In January to March 2015, a total of 459,700 Europeans, including 12,600 from the Netherlands, had travelled to Australia [13]. Infection is preventable using common mosquito-prevention measures such as wearing long trousers, long sleeves, light-coloured clothes and insect repellents.

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Tessa Bal, Sandra Scherbeijn, Sabine Köhler, Birgit Hüsing, Insa Bonow, Alexandra Bialonski, Mathis Petersen are acknowledged for excellent technical assistance. Annemiek van der Eijk for discussions and critical reading of the manuscript.

Conflict of interest
None declared.

Authors' contributions
Chantal Reusken: coordination, data analysis, wrote article. Natalie Cleton: data analysis. Mariana Medonça Melo: treating physician. Chantal Visser: treating physician. Corine Geurts van Kessel: case medical microbiologist. Peter Bloembergen: case medical microbiologist. Marion Koopmans: co-wrote article. Jonas Schmidt-Chanasit: reference testing, co-wrote article. Perry van Genderen: treating physician, co-wrote article.

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Spot the Difference: Development of a syndrome based protein microarray for specific serological detection of multiple flavivirus infections in travelers.

Cleton N., Godeke GJ., Reimerink J., Beersma M., Doorn R., Franco L., Goeijenbier M., Jimeneze-Clavero M., Johnson B., Niedrig M., Papa A., Sambri V., Tami A., Velasco-Salas Z., Koopmans M. and Reusken C.

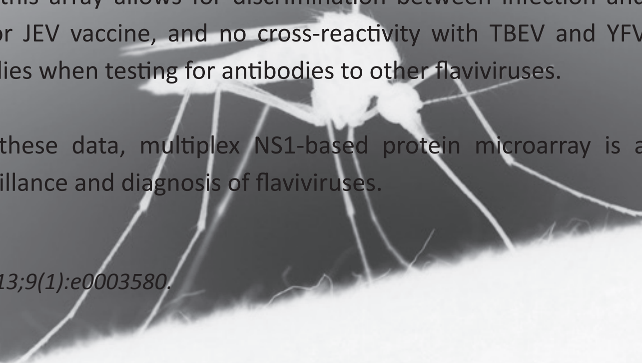
Abstract

Background: The family *Flaviviridae*, genus *Flavivirus*, holds many of the world's most prevalent arboviral diseases that are also considered the most important travel related arboviral infections. In most cases, flavivirus diagnosis in travelers is primarily based on serology as viremia is often low and typically has already been reduced to undetectable levels when symptoms set in and patients seek medical attention. Serological differentiation between flaviviruses and the false-positive results caused by vaccination and cross-reactivity among the different species, are problematic for surveillance and diagnostics of flaviviruses. Their partially overlapping geographic distribution and symptoms, combined with increase in travel, and preexisting antibodies due to flavivirus vaccinations, expand the need for rapid and reliable multiplex diagnostic tests to supplement currently used methods.

Goal: We describe the development of a multiplex serological protein microarray using recombinant NS1 proteins for detection of medically important viruses within the genus *Flavivirus*. Sera from clinical flavivirus patients were used for primary development of the protein microarray.

Results: Results show a high IgG and IgM sensitivity and specificity for individual NS1 antigens, and limited cross reactivity, even within serocomplexes. In addition, the serology based on this array allows for discrimination between infection and vaccination response for JEV vaccine, and no cross-reactivity with TBEV and YFV vaccine induced antibodies when testing for antibodies to other flaviviruses.

Conclusion: Based on these data, multiplex NS1-based protein microarray is a promising tool for surveillance and diagnosis of flaviviruses.



PLoS Negl Trop Dis. 2015 Mar 13;9(1):e0003580.

Introduction

The family *Flaviviridae*, genus *Flavivirus*, holds many of the world's most prevalent arboviral diseases that are also considered the most important travel related arboviral infections.[1] As the geographic distribution and symptoms caused by these viruses overlap, detection requires differential diagnostic algorithms that include multiple flaviviruses.[2] Increase in travel expands the need for rapid and reliable multiplex diagnostic tests in non-endemic countries to supplement currently used methods.[3, 4]

Flaviviruses are single stranded enveloped viruses with an RNA genome of about 11 kb length. The genome is composed of three structural (Envelope, Capsid and Precursor-membrane) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5).[5] Diagnosis is primarily based on serology through detection of IgM and IgG antibodies, as viremia typically has been reduced to undetectable levels when symptoms set in and patients seek medical attention.[5-7]

The genus is divided into serocomplexes that are distinguished based on neutralizing antibody reactivity (Figure 1). The amino acid homology of the envelope (E) protein (which is the immunodominant antigen for neutralizing antibody assays) ranges from 40-50% between serocomplexes and 70-80% for virus species within a serocomplex.[5, 8] Antibodies to flaviviruses are known to cross-react extensively within, and to a certain extent between, serocomplexes when using traditional antibody assays.[9-12] Cross-reactivity occurs also if patients have been vaccinated against flaviviruses such as yellow fever virus (YFV), tick-borne encephalitis virus (TBEV) and/or Japanese encephalitis virus (JEV) or after secondary infection with a different flavivirus.[9, 10, 13]

To overcome flavivirus cross-reactivity in diagnostics the use of recombinant antigens in ELISA is to be preferred over whole virus as it increases specificity.[14-16] Envelope, pre M and NS1 recombinant proteins are the most commonly used.[14-16] Of these, the NS1 has shown to be highly immunogenic and important in the development of non-neutralizing protective antibodies.[17, 18] NS1 is thought to contain more species specific epitopes than the envelope protein, although some cross-reactivity is seen between NS1 proteins.[19-21] NS1 in its natural conformation is thought to elicit a more specific immune response.[22, 23] The absence of NS1 proteins in inactivated JEV vaccines offers further potential for serological diagnosis through allowing differentiation between vaccinated and infected patients.[24] Thus, NS1 protein shows potential to use in serological differentiation between flavivirus infections.[25, 26]

To enable fast, syndrome based laboratory testing that focuses on multiple rather than individual viruses, we developed a protein microarray, using recombinant NS1 proteins, as a serological test for medically important viruses within the *Flavivirus* genus.

Materials and Methods

Samples

Sera from anonymized patients were used for primary development of the protein microarray. Patients were diagnosed according to international accepted criteria combining clinical symptoms, epidemiological data, and standard serological methods (ELISA, IFA) and laboratory confirmed by either VNT or PCR with the exception of 10 patients suspected of JEV. Information on each patient group used is presented in Table 1.

Protein production

Custom-made NS1 proteins produced in human embryonic kidney 293 (HEK293) cells to ensure proper folding, glycosylation and dimerization were used (Immune Technology Inc., New York, NY, USA). A V5-epitope and Histag were added to the C-terminus for protein quantification and filtration. Proteins were expressed for Dengue virus 1 (genbank:FJ687432.1), Dengue virus 2 (genbank:FJ744720.1), Dengue virus 3, (genbank:FJ744738.1), Dengue virus 4 (genbank:EU854300.1), Japanese encephalitis virus (genbank:NC_001437.1), St. Louis encephalitis virus (genbank:ACB58159.1), Yellow fever virus (genbank:JN620362.1) and West Nile virus (genbank:EU081844.1)

Usutu virus NS1 (genbank:NC006551.1) was produced in-house in a HEK293 cell-line. The NS1 gene was produced by Genscript (NJ, USA) with and additional V5-epitope and Histag on the C-terminus and cloned into a pcDNA-DEST40 vector (Invitrogen, Thermo Fisher Scientific, MA, USA) that contained a neomycine resistance gene. The vector which contained the NS1 gene was transfected into HEK293 cells. Neomycine resistant clones were selected and tested for protein expression by immune fluorescent assay using anti-V5 monoclonal antibody. Selected clones were grown in flasks and secreted NS1 protein into the medium (Opti-MEM, Thermo Fisher Scientific, MA, USA). The secreted protein was purified from the medium by FPLC using a Ni-NTA column (Qiagen, CA, USA) according to the manufacturer's instructions.

Microarray slide preparation

NS1 antigens at concentrations of around 2mg/ml were mixed with protein arraying buffer (Maine manufacturing, GVS Group, Italy) and spotted in triplicate as a within-test control per pad. Antigens were spotted onto a nitrocellulose pad coated glass slide (Maine manufacturing, GVS Group, Italy) using a non-contact protein array spotter (PerkinElmer, Waltham, MA, USA) as previously described.[27] Per spot two drops of 333 pL of diluted protein were used. After printing, slides were placed in a drying chamber overnight and stored at room temperature until use.

Protocol testing for IgG and IgM antibodies

Patient sera were tested on dried slides as previously described.[27] In short, slides were incubated in Blotto blocking-buffer (Thermo Fisher Scientific, MA, USA) for one hour at 37° C in an incubation chamber to reduce non-specific binding of serum. Serum was diluted in eight two-fold dilution steps (1:10 to 1:2560) in blotto supplemented with 0.1% Surfact-Amp (Thermo Fisher Scientific, MA, USA) and incubated for 1 hour at 37° C in a moist chamber. Incubation followed with an Fc-fragment specific IgG or Fc5μ-fragment specific IgM specific conjugate with a Cy5-fluorescent dye (Invitrogen, California, USA) for one hour at 37°C. For IgM detection, serum was first depleted of IgG antibodies using Gullsorb (Meridian Bioscience, OH, USA) according to the manufacturer's instructions. Between each incubation step, slides were washed three times with a protein array washing buffer (Thermo Fisher Scientifc, Rockford, MA, USA). After final wash, slides were scanned with a Tecan scanner (Tecan Trading AG, Männedorf, Switzerland). A median fluorescence signal (measured at 647nm) for each of the triplet spots per antigen was determined by ScanArray Express 4.0.0.0001 supporting program (PerkinElmer, MA, USA) using an adaptive circle (diameter 80–200 μm). The fluorescent signal ranged from 0 to a maximum of 65,536 units. Results were imported in R for analysis.[28]

Table 1: Overview serum collection used for flavivirus microarray development

Virus species*	County of origin	Number of samples	Days post onset symptoms	PCR confirmed	Virus neutralization confirmed (VNT/PRNT)	Serology (ELISA/IFA/ Luminex)
DENV1-2	Vietnam: Hospital for Tropical Diseases, Ho Chi Minh City, Vietnam	19	Hospitalized patients 2-7 days post onset symptoms	19/19	0/19	19/19
DENV1-4	Venezuela: Carabobo University, Faculty of Science and Technology, Department of Biology, Venezuela	12	3-21 days post onset symptoms	12/12	0/12	12/12
DENV1-3	Spain: National Centre for Microbiology. Instituto de Salud Carlos III.,Madrid, Spain	27	1-17 days post onset symptoms with travel history	27/27 (PCR or NS1-capture)	0/27	27/27
WNV	Greece: Department of Microbiology, Medical School, Aristotle University of Thessaloniki, Greece	7	9-23 days post onset symptoms	0/7	7/7	7/7
WNV	Netherlands: National Institute for Public Health and Environment, The Netherlands	5	5-21 days post onset symptoms with travel history	0/5	5/5	5/5
2xWNV; 1x SLEV; 1x YFV-vac	USA: US Centers for Disease Control and Prevention, Division of Vector-Borne Diseases, Arbovirus diagnostic and reference laboratory	4	Samples were part of the CDC 2011 reference panel for WNV serology	0/4	4/4	4/4
JEV	Vietnam: Hospital for Tropical Diseases, Ho Chi Minh City, Vietnam	10	From hospilized patients with acute encephalitis 6-18 days post onset symptoms	0/10	0/10	10/10: serially tested by two independent tests (ELISA and IFA) at two independent laboratories**
1x JEV; 1x YFV	Netherlands: National Institute for Public Health and Environment & Erasmus Medical Centre, The Netherlands	2	From hospitalized clinical patients 5-10 post onset symptoms with travel history	1(YFV)/2	2/2	2/2

1x pooled USUV	Centro de Investigación en Sanidad Animal, Madrid, Spain	1	Pooled rabbit sample 14 days post infection	1 /1	1/1	No tests available
2x human USUV	DIMES - University of Bologna, Unit of Microbiology, Italy	2	The only two human encephalitis cases reported in Europe	0/2	2/2	No tests available
Base-line group	The Netherlands: National Institute for Public Health and Environment	85	Dutch blood donors with unknown travel history and vaccination history	0/85	0/85	85/85: without detectable antibodies to WNV, DENV or TBEV
Vaccinated group	The Netherlands: National Institute for Public Health and Environment & Erasmus Medical Centre, The Netherlands and Germany: Centre for Biological Threats and Special Pathogens, Robert Koch-Institut, Germany	23	Vaccinated individuals with proven YFV, TBEV and/or JEV IgG titers	0/23	19/23	23/23
1x pooled JEV/DENV negative control; 1x pooled DENV1-4 positive control; 1x pooled post-JEV-vac	UK: NIBSC National Institute for Biological Standards and Control, UK	3	International reference samples: reference number #01/184, #01/186, #01/182	3/3	3/3	3/3

* DENV1-4 = Dengue virus serotype 1 to 4; JEV = Japanese encephalitis virus; SLEV = St. Louis encephalitis virus; TBEV-vac = Tick-borne encephalitis vaccinated; USUV = Usutu virus; WNV = West Nile virus; YFV = Yellow fever virus; YFV-vac = Yellow fever virus vaccinated;
** Hospital for Tropical Diseases, Ho Chi Minh City, Vietnam and National Institute for Public Health and Environment, the Netherlands

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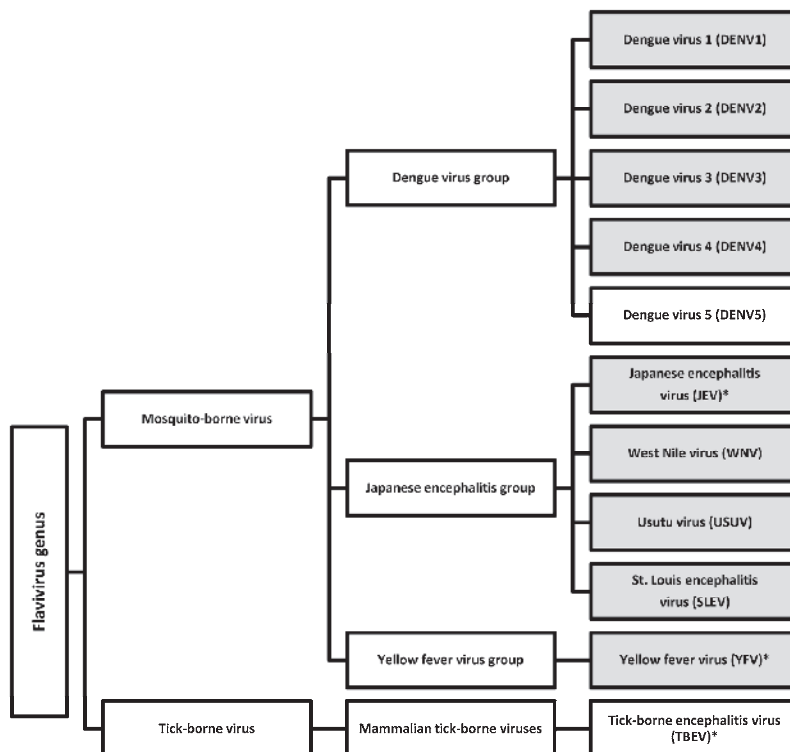
Figure 1: The serogroup classification of the *Flavivirus* genus of arboviruses used. Shaded boxes indicate antigens and antibodies used in this validation. Viruses with an * indicate that human vaccines are available for this virus.



Protein concentration optimization

Virus antigens were spotted in serial two-fold dilutions ranging from 1:2 to 1:16 for initial checkerboard titrations to determine optimum protein concentration as previously described.[27] Antigens were tested using serially diluted anti-V5-epitope monoclonal antibodies (Invitrogen, Thermo Fisher Scientific, Rockford, MA, USA). Optimum protein concentrations were defined as those at which maximum fluorescent signal and overlapping s-curves were achieved for anti-V5-epitope monoclonal antibodies and were found to be around 0.5 mg/ml. To minimize batch-to-batch variations each batch was tested with a serial dilution of anti-V5 monoclonal antibodies if a more than 10% variation was found in reference to the initial test batch the slides were excluded. Day-to-day variations were monitored by including a positive and negative WHO DENV1-4 reference serum during each test round. If a more than one titer dilution-step difference was detected results were excluded.[29]

Analysis

A script was written in R[28] using additional package ‘drc’ version 2.3-7[30], as previously described.[27] The median fluorescent signals were converted into fitted dilution-s-curves per protein for each serum sample. Additional script was written that allowed titers to be

calculated on the estimated s-curve at a given ROC calculated cut-off. Optimal signal cut-offs were determined by a log2 transformation of signals to further reduce variance caused by day-to-day and slide-to-slide variations. Optimal signal cut-offs were achieved by selecting the highest possible combination of sensitivity and specificity through ROC optimal curve calculations performed in GraphPad Prism.[31] Titers were defined as the highest serum dilution with a signal above the cut-off determined by ROC analysis. Heat maps were generated using an additional R package 'stats'[28] and based on pairwise correlation between rows and columns. Mann-Whitney tests were employed to establish the significance of differences between groups.

Results

Sensitivity and specificity for individual antigens

The mean antigen reactivity by NS1 proteins in 1:10 to 1:80 start dilutions was high in homologous DENV, WNV, JEV, SLEV, YFV and Usutu virus (USUV) positive control sera and low in negative control sera and in sera from individuals vaccinated for JEV, TBEV or YFV ($p<0.01$) with the exception of YFV NS1 antigen with YFV vaccinees (Figure 2 and Table 2). Only some NS1 reactivity was observed in samples from blood donors for other antigens (1%).

At low serum dilutions, some patients showed antibody IgG reactivity to multiple antigens, and therefore ROC curves were calculated in multiple dilutions and the signals for the 1:20 dilutions were used for signal cut-off calculations. The 1:10 and 1:20 serum dilutions produced comparable results in sensitivity and specificity, but with significantly lower background for the 1:20 dilutions. At 1:40 serum dilutions, the sensitivity started to decrease.

Only 13 DENV positive patients (travelers) had known primary DENV infections with a PCR confirmed serotype (DENV1-3). All other patients with PCR confirmed DENV (serotype 1-4) were from DENV endemic countries and could not be confirmed as primary infections. As not all DENV infections were known to be primary, the highest signal to DENV1-4 NS1 was used for calculation of the DENV cut-offs. The optimal cut-off for all proteins was around a fluorescent signal of 15,000 for IgG and 4,000 for IgM, producing sensitivity and specificity of 86% to 100% and 86% to 100% respectively (Table 3).

For USUV, SLEV and YFV only one or two positive patient samples were available so that proper ROC curves could not be calculated, but background signals were in the same range as for the other antigens (Table 2). Serum samples from YFV-vaccinees were strongly positive for YFV. Some blood donors had YFV signals above the cut-off, probably reflecting vaccination history (Figure 2).

Cross-reactivity

In order to study cross-reactivity within and between serocomplexes, serum samples were serially diluted and titers were calculated in R. Typical individual patient profiles are shown in figure 3. To quantify the degree of cross-reactivity, the ratio of the signal for each antigen to the maximum signal measured for that serum (typically the homologous antigen) was calculated (Figure 4a-c). With one exception for IgG (serum sample #4), all patients had the highest IgG and IgM reactivity with the homologous NS1 antigen. High level IgG reactivity to a second antigen was observed for two of the DENV patients (against WNV and JEV, respectively) and for 2 JEV patients (against DENV) (Figure 4a). One serum sample from a JEV

patient (serum sample 4) had a higher titer DENV NS1 in comparison to JEV NS1. For IgM, only homologous reactivity was observed.

Serotype specific reactivity for DENV
IgG profiles from individual patients were combined into a heatmap (Figure 5) to confirm grouping according to exposure history. One group of patients (indicated by a star in the heatmap) showed high titers to multiple DENV serotypes. A larger group had highest titers to a single DENV serotype, suggesting serotype specificity of the antibody array results. As most patients were from different regions, the data were stratified for non-endemic (travelers) and multiple DENV endemic countries. This showed a significant difference in titers between groups ($p<0.01$) for IgG but not for IgM ($p=0.25$) titers.
For 13 known primary DENV cases, the serotype had been determined by RT-PCR. All but one serum had highest IgG antibody levels to the infecting serotype, but IgM antibody reactivity was lower and less discriminatory.

Discussion
We developed a first generation protein microarray for rapid, multiplex and virus-specific IgM and IgG tests for diagnosis of flavivirus infections that could differentiate between virus species, even within flavivirus serocomplexes, as well as between vaccinated and infected individuals, with the exception of YFV vaccinated individuals. Initial validation shows that this microarray can be used for flavivirus surveillance in travelers and potentially in regions with co-circulation of multiple flaviviruses. To establish this we first investigated the sensitivity of NS1 antigens to their homologous sera on a protein microarray. The results show good IgG and IgM sensitivity for both JEV and DENV serocomplex viruses. The sensitivity is comparable to current IFA and ELISA commercial kits. However, our sample selection was tested with a multitude of standard serological assays. Comparing our results to commercial ELISA or IFA kits should therefore be done with caution. Our results confirm that NS1 provides a good sensitive and specific antigen tool for serological diagnosis.

For DENV, the sensitivity of the IgM assay was lower than for IgG. There are several possible explanations for this finding. First, lower DENV IgM sensitivity may have resulted because five to ten days post onset of symptoms may be too early for detecting seroconversion in serum samples.[32] Evaluations of commercial flavivirus IgM diagnostics kits showed varying sensitivities ranging from 58% to 98%, partially due to sample timing.[32-34] Five days post onset of symptoms, around 50-80% of patients on average have detectable IgM antibodies. This increases to 99% 10 days post onset of symptoms.[35]
Second, a study of antibody reactivity to individual DENV proteins has found that the measured mean OD is lower for NS1 compared to E-protein with many samples clustering closer to the cut-off compared to E-protein antibodies.[18] This makes NS1 antibody detection more susceptible to timing of sample taking and detection limit of test used.
Third, patient-to-patient variation in antibody responses to individual viral antigens may cause discrepancies of test results. NS1 based protein assays could potentially pick up infections missed by prM-E based front-line serological tests.[20] Antibodies to envelope protein were detected in 91% of the DENV cases while NS1 antibodies were detected in 99%, indicating that NS1 has a higher sensitivity.[18] Two of the nine DENV IgM positive samples from travelers that tested negative on DENV IgG ELISA, which is based on the prM-E antigen, tested IgG positive on our microarray, further supporting this assumption.

Figure 2: IgG fluorescent intensity (measured at 647nm) to flaviviruses in serum samples from clinical patients, persons vaccinated with YFV, JEV or TBEV and healthy blood donors in a 1:20 serum dilution. NS1 proteins were spotted in a 0,5mg/ml concentration. Y axis represents the fluorescent intensity. The median signal is depicted as a line. The dotted line represents the calculated cut-off by the ROC used to determine antibody titers.

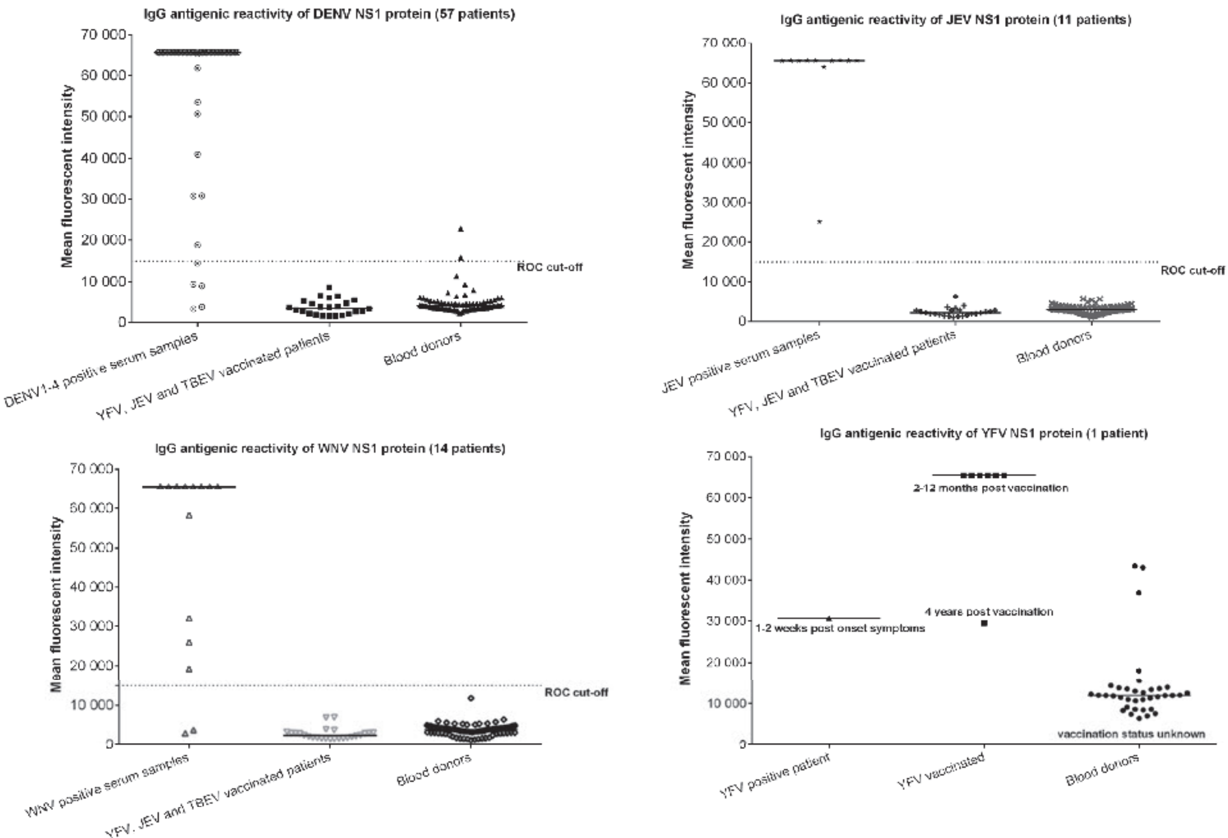


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Figure 3: IgM fluorescent intensity (measured at 647nm) to flaviviruses in serum samples from clinical patients, persons vaccinated with YFV, JEV or TBEV and healthy blood donors in a 1:20 serum dilution. NS1 proteins were spotted in a 0,5mg/ml concentration. Y-axis represents the fluorescent intensity. The median signal is depicted as a line. The dotted line represents the calculated cut-off by the ROC used to determine antibody titers.

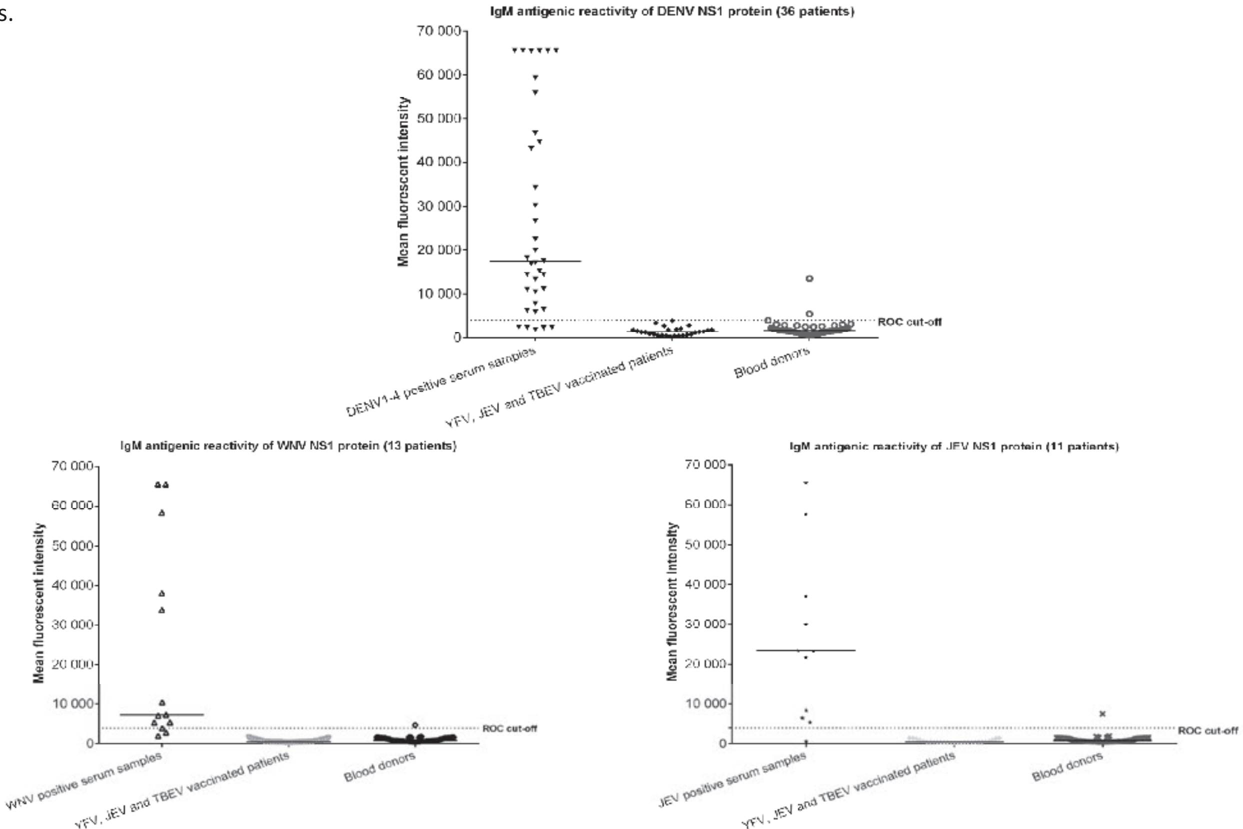

Figure 4: Representative examples of IgG antibody profiles of individual patients infected with JEV (Figure 3A) and DENV (Figure 3B) serocomplex viruses.

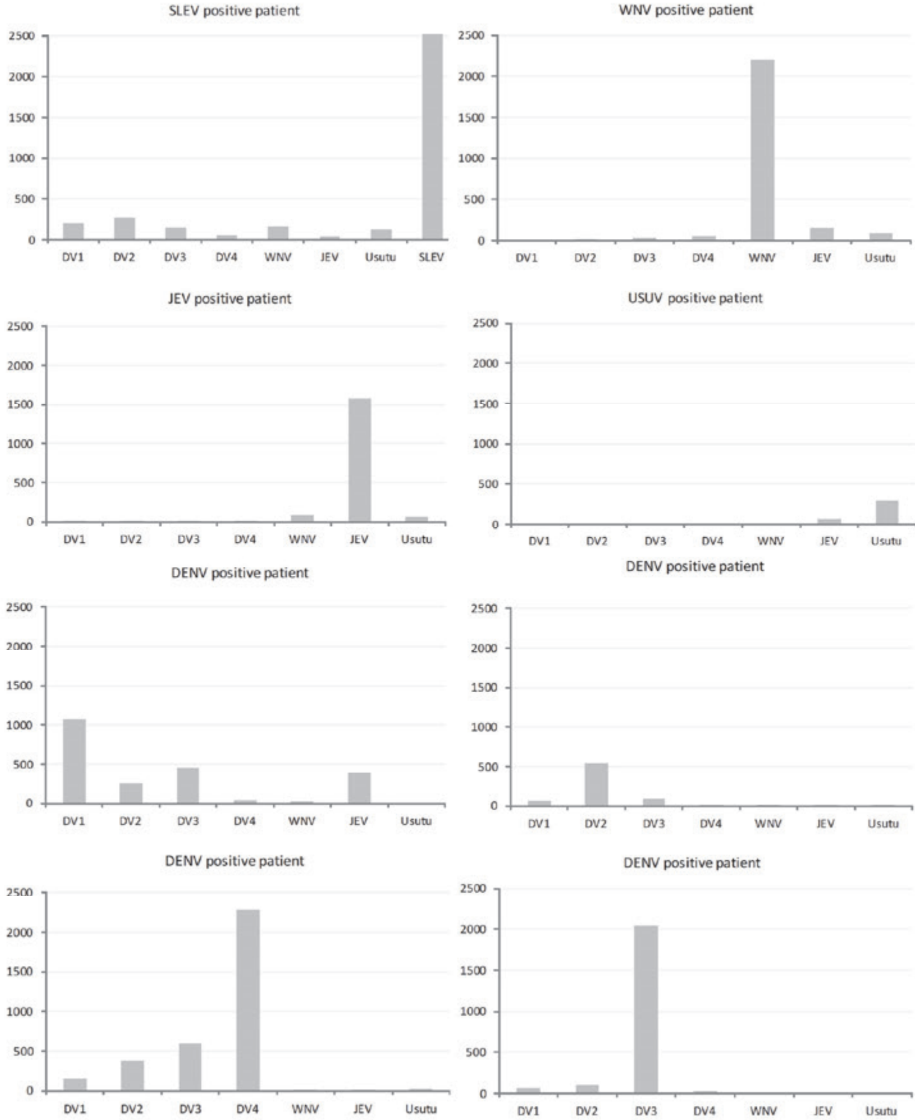

Figure 5: Analysis of cross-reactivity for microarray based flavivirus serology. To visualize the cross-reactivity seen in individual serum samples the maximum calculated titer per sample was set at 100% and all other signals were expressed as a percentage of the highest titer (0-100% on y-axis).

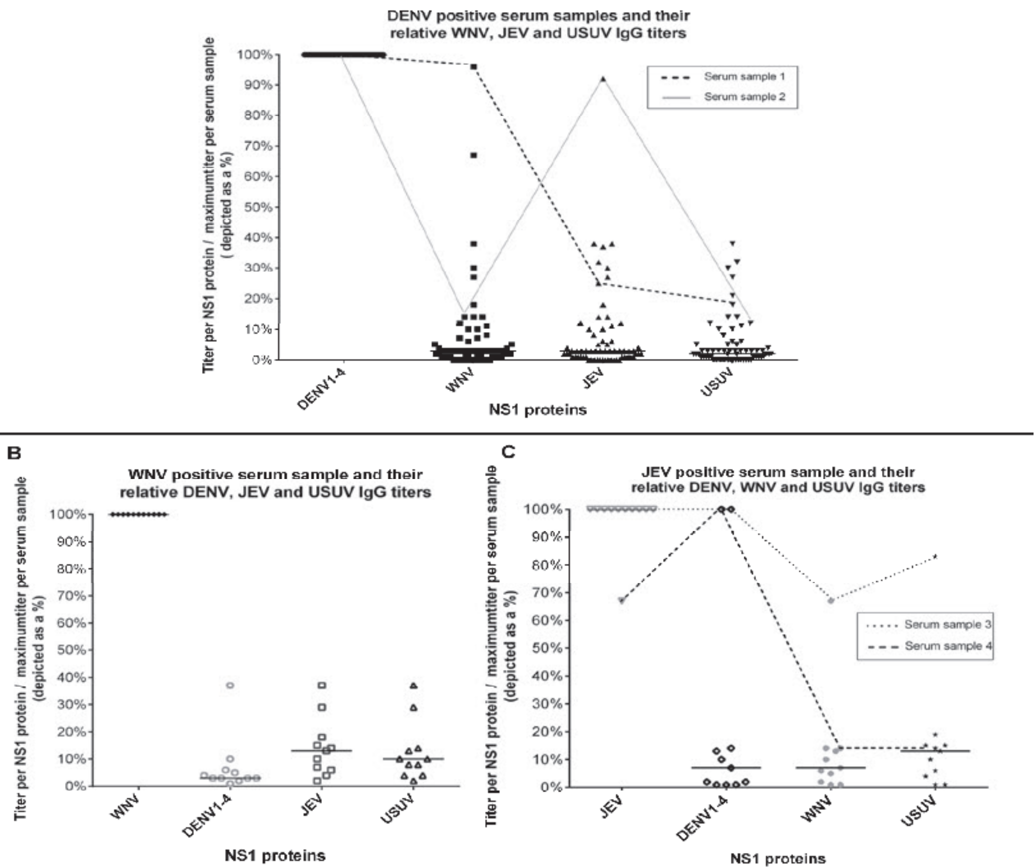

Figure 6: Heatmap of patient IgG antibody profiles. To visualize the overall cross-reactivity seen in individual serum samples the maximum calculated titer per sample was set at 100% and all other signals were expressed as a percentage of the highest titer and placed in a heatmap. White refers to a titer of 0% in reference to highest calculated titer per serum sample with a sliding scale to red which indicates a titer of 100% comparable to highest titer calculated. The numbers alongside the patient group column correspond to the serum samples shown in figure 4A and 4C. The star indicates a group of patients with high titers to multiple DENV NS1 antigens.

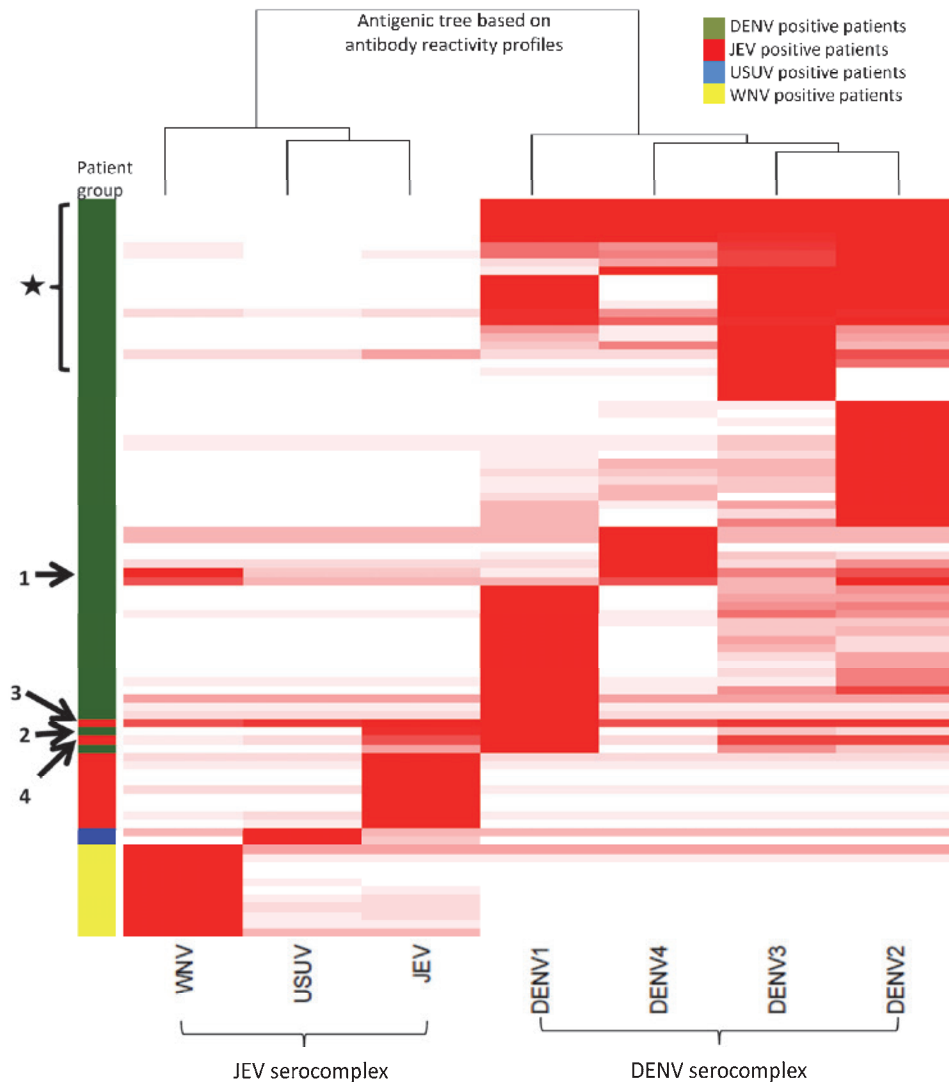

Table 2: Median signal and 25-75% percentile for IgG and IgM per virus antigen test group.

Serum dilution	1:20 IgG		1:20 IgM			1:20 IgG		1:20 IgM	
VIRUS	**Median**	**25-75% percentile**	**Median**	**25-75% percentile**	**CONTROL**	**Median**	**25-75% percentile**	**Median**	**25-75% percentile**
Dengue	65,535	65,535	17,421	8,487-46,244	Vaccinated	3,440	2,105-5,070	1,387	536-1,876
					Blood donors	4,057	3,350-4,880	1,632	1,181-1,984
West Nile	65,535	24,350-65,535	7,311	4,678-48,174	Vaccinated	2,382	1,742-3,026	542	478-972
					Blood donors	3,861	3,025-4,471	817	591-1,456
Japanese encephalitis	65,535	65,535-65,535	23,364	65,04-37,139	Vaccinated	2,138	1,559-2,792	529	410-921
					Blood donors	2,945	2,368-3,563	806	568-1,527
Usutu	43,939	22,342-65,535	NA	NA	Vaccinated	2,804	1,961-3,101	ND	ND
					Blood donors	3,382	2,778-4,008	809	594-1,848
Yellow fever	30,680	30,680-30,680	NA	NA	Vaccinated	65,535	6,5535-6,5535	NA	NA
					Blood donors	11,925	9,497-13,576	3,163	1,346-4,160
St. Louis encephalitis	65,535	65,535-65,535	NA	NA	Vaccinated	ND	NA	NA	NA
					Blood donors	4,808	3,636-5,649	878	711-1,084

ND = Not done; NA = Not available; Vaccinated = Vaccinated for TBEV, YFV and/or JEV

Table 3: Sensitivity and specificity for each virus antigen group with 95% CI. The number of positive samples are indicated in column 'n samples' and the number of negative samples are shown behind brackets in the 'Spec' column.

Group	n samples	Sens IgG	Spec IgG (n82)	n samples	Sens IgM	Spec IgM (n80)
DENV	57	0.92 (0.93-0.98)	0.99 (0.95-1.00)	36	0.86 (0.71-0.95)	0.98 (0.93-1.00)
WNV	14	0.86 (0.57-0.98)	1.00 (0.97-1.00)	13	0.85 (0.55-0.98)	0.99 (0.95-1.00)
JEV	11	1.00 (0.72-1.00)	1.00 (0.97-1.00)	11	0.91 (0.59-1.00)	0.99 (0.95-1.00)

n samples = number of positive samples used; Sens= sensitivity; Spec = specificity

Finally, a number of the DENV cases may have been secondary, tertiary or quaternary infections. Several of the samples originated from DENV hyperendemic countries and showed high titers to multiple DENV and other flaviviruses, further supporting this assumption. Literature shows that IgM antibody titers produced during a secondary DENV infection are absent or lower and produced during a shorter period. This reduces the sensitivity of IgM antibodies in non-primary infections significantly.[35] Despite the above limitations, the NS1-based protein microarray performed well. Ideally, further validation is needed with sequentially sampled patient sera, allowing evaluation optimization of timing of sampling during infection, in relation to cut-off chosen.

The biggest challenge of current flavivirus serodiagnostics is virus specific differentiation.[3, 4] We analyzed the signals produced towards all antigens per sample to test our protein microarray's capability to achieve this. With three exceptions (probable secondary infections), clear differentiation of antibody responses to the homologous antigen (defined as the virus the patient was confirmed infected with) was found for most patients. This can be clearly seen in the heatmap (Figure 5). This is a big advantage over currently available serological tests, for which particularly flavivirus vaccination causes extensive cross-reactivity, mainly for IgG.[9-12] Previous epitope analysis of DENV NS1 and envelope protein of indicated that NS1 has more virus specific epitopes and thus could be used for more specific serological tests.[16, 19-22] Our results support this showing good specificity for the NS1-based protein microarray.

The cause of the outliers shown in figure 4 is unclear as they may be the result of cross-reactive antibodies or previous exposure. Cross-reactive antibodies to NS1 proteins have been detected in previous studies, but why they are seen in some patients and not others remains unclear.[20] The reactivity to multiple antigens more likely reflects differences in exposure history.[10, 36] This assumption is supported by the fact that most patients with reactivity to multiple antigens originated from countries where multiple flaviviruses are endemic (Vietnam and Venezuela). The IgG titers of these patients are log multiplications higher than singular reactive titers and their corresponding IgM titers, which is highly indicative for secondary and frequent flavivirus infections.[16] Distinguishing primary from secondary or multiple flavivirus infections is serologically difficult.[16] Further investigation into patients with known multiple flavivirus infections will be needed to further define the uses of our multiplex array testing in such patient populations.

While we show excellent discrimination in antibody responses to viruses within the JEV complex, this is less straightforward for DENV. The protein microarray IgG antibody reactivity to individual DENV NS1 antigens shows capability to distinguish serotypes (as can be seen in figure 5), but this is not seen to the same extent for IgM antibodies. This was surprising as flavivirus IgM envelope antibodies are thought to be more specific than IgG antibodies.[16] However, to what extent this can also be said for NS1 is unclear. Here, future work will focus on more extensive evaluation, for which well characterized patient cohorts are needed.

Finally, we looked at the ability to rule out false positive reactions due to vaccination. This was not possible for YFV vaccination when testing against YFV NS1. YFV vaccine is a live attenuated vaccine and causes mild infection resulting in NS1 antigen and antibody production.[37] We do, however, show that when using NS1 antigens the cross-reactivity

between the YFV serocomplex and other serocomplexes is absent.[9, 10, 13] JEV vaccine currently on the European and American market is an inactivated whole virus vaccine based on JEV virions.[38] This production technique makes NS1 a good target for vaccination versus infection differentiation and is the basis for current surveillance programs in Japan and surrounding countries.[39] However, live-attenuated (non-recombinant) JEV SA-14-14-2 vaccine is currently in use in a number of countries in Asia and has recently been WHO prequalified. Its use nullifies the ability to differentiate vaccination from infection, reducing Public Health surveillance options. New vaccine methods using chimeric virus vaccines are in development and might provide new opportunities for vaccination and infection differentiation through NS1. In veterinary vaccine development, good practice ensures the ability for such discrimination through the development of marker vaccines according to the DIVA (Differentiating Infected from Vaccinated Animals) principle.[40] Public health challenges associated with inability to reliably provide patient diagnostics in some instances due to vaccination, highlight the need for introducing this principle to the human vaccine market.

Conclusion

Serological differentiation between flaviviruses and the false-positive results caused by vaccination are a serious problem for surveillance and diagnostics of flaviviruses. Analysis of our NS1-based protein microarray results showed a high IgG and IgM sensitivity and specificity for individual antigens even within the same serocomplex, and limited cross reactivity. In addition, the serology based on this array allowed discrimination between infection and vaccination response for JEV vaccine, and no cross-reactivity with TBEV and YFV vaccine induced antibodies when testing for antibodies to other flaviviruses. Based on this data, our multiplex NS1-based protein microarray is a promising tool for surveillance and diagnosis of flaviviruses.

Acknowledgements

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Accession numbers of NS1 proteins used:

Dengue virus 1 (genbank:FJ687432.1), Dengue virus 2 (genbank:FJ744720.1), Dengue virus 3, (genbank:FJ744738.1), Dengue virus 4 (genbank:EU854300.1), Japanese encephalitis virus (genbank:NC_001437.1), St. Louis encephalitis virus (genbank:ACB58159.1), Yellow fever virus (genbank:JN620362.1) and West Nile virus (genbank:EU081844.1), Usutu virus NS1 (genbank:NC006551.1).

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5.2 |

5.2

A serological protein microarray for detection of multiple cross-reactive flavivirus infections in horses for veterinary and public health surveillance.

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Abstract

Background: The genus *Flavivirus* in the family *Flaviviridae* includes some of the most important examples of emerging zoonotic arboviruses that are rapidly spreading across the globe. Japanese encephalitis virus (JEV), West Nile virus (WNV), St. Louis encephalitis virus (SLEV) and Usutu virus (USUV) are mosquito-borne members of the JEV serological group. Although most infections in humans are asymptomatic or present with mild flu-like symptoms, clinical manifestations of JEV, WNV, SLEV, USUV and Tick-borne encephalitis virus (TBEV) can include severe neurologic disease and death. In horses, infection with WNV and JEV can lead to severe neurological disease and death while USUV, SLEV and TBEV infections are mainly asymptomatic, however induce antibody responses. Horses often serve as sentinels to monitor active virus circulation in serological surveillance programs specifically for WNV, USUV and JEV.

Methods: Here we developed and validated a NS1-antigen protein microarray for the serological differential diagnosis of flavivirus infections in horses using sera of experimentally and naturally infected symptomatic as well as asymptomatic horses.

Results: Using samples from experimentally infected horses, an IgG and IgM specificity of 100% and a sensitivity of 95% for WNV and 100% for JEV was achieved with a cut-off titre of 1:20 based on ROC-calculation. In field settings, the microarray identified 93-100% of IgG positive horses with recent WNV infections and 87% of TBEV IgG positive horses. WNV IgM sensitivity was 80%. Differentiation between closely related flaviviruses by the NS1-antigen protein microarray is possible, even though we identified some instances of cross-reactivity among antibodies. However, the assay is not able to differentiate between naturally infected horses and animals vaccinated with an inactivated WNV whole-virus vaccine.

Conclusion: We showed that the NS1-microarray can potentially be used for diagnosing and distinguishing flavivirus infections in horses, and for public health purposes within a surveillance setting. This allows for fast, cheap, syndrome-based laboratory testing for multiple viruses simultaneously for veterinary and public health purposes.

Submitted

Introduction

Arboviruses (arthropod-borne viruses) are considered emerging pathogens because of their increasing incidence and geographical expansion to previously unaffected areas.[1] The emergence of arboviral diseases depends on many socio-economic, environmental and ecological factors, including vector and host population dynamics, travel and trade, and climate change.[2] Recent outbreaks have demonstrated that the emergence of arboviruses can have severe consequences for both public and veterinary health.[3, 4]

The genus *Flavivirus* in the family *Flaviviridae* provides some of the most important examples of emerging arboviruses that are rapidly spreading across the globe. A recent example, the rapid spread of Zika virus within the America's, urged the World Health Organisation (WHO) to declare the outbreak of Zika virus associated microcephaly a Public Health Emergency of International Concern. Japanese encephalitis virus (JEV), West Nile virus (WNV), St. Louis encephalitis virus (SLEV) and Usutu virus (USUV) are also mosquito-borne members of the JEV serogroup.[5]

These closely related flaviviruses are maintained in an enzootic transmission cycle involving birds as the main reservoir hosts.[6] Tick-borne encephalitis virus (TBEV) is a member of the tick-borne encephalitis group and is sustained in a rodent-tick transmission cycle. To a great extent, the geographical ranges of these five viruses overlap. Humans and horses are incidental, dead-end hosts.[7] Although most infections in humans are asymptomatic or present with mild flu-like symptoms, clinical manifestations of JEV, WNV, SLEV, TBEV and USUV can include severe neurologic disease and death.[8] In horses, infection with WNV and JEV can lead to severe neurological disease and death while USUV, SLEV and TBEV infection are mainly asymptomatic but do induce antibody responses.[9] Horses often serve as sentinels for active virus circulation in serological surveillance programs specifically for WNV, USUV and JEV.[10-13]

Flavivirus infections can be diagnosed by serology, virus isolation, or molecular techniques.[14] The latter two methods are often unsuccessful because of reduced viremia at the time of symptom presentation in dead-end hosts.[15] Diagnosis is therefore primarily based on the detection of antibodies directed against flaviviruses in serum or, in case of neurological manifestation, in cerebrospinal fluid (CSF) of infected individuals.[7] Frequently used serological tests such as enzyme-linked immunosorbent assay (ELISA) and immuno-fluorescence assay (IFA) are mainly focused on the detection of antibodies directed against the whole virus or flavivirus envelope (E) protein, to which the majority of the antibody response is targeted.[16] A major limitation of these serological tests is the presence of extensive IgG, and to some extent IgM, antigenic cross-reactivity among viruses of the same serogroup and even between serogroups.[17] Immunity due to previous flavivirus infection or vaccination can complicate the diagnosis of recent infections.[18] Human vaccines exist for JEV, yellow fever virus (YFV), dengue virus (DENV, only approved for use in Mexico) and TBEV; equine vaccines have been widely deployed for JEV and WNV.

To enable identification of potential flavivirus threats, new diagnostic procedures and specific serological tests are required that focus on the simultaneous but specific detection of multiple instead of individual pathogens or antibodies against these pathogens.[19] Protein microarrays provide such an alternative high-throughput diagnostic system.[20]

Previously, we developed a protein microarray for detection of antibodies to flaviviral non-structural protein 1 (NS1) in returning travellers, using sera from clinical flavivirus patients.[21] Building on that microarray, here we developed and validated a protein microarray for the differential diagnosis of flavivirus infections in horses using sera of clinical and non-clinical flavivirus infected horses. This allows for fast, less expensive, syndrome-based laboratory testing for multiple viruses simultaneously for veterinary and public health purposes.

Material and methods

Serum cohorts

Different serum cohorts were used in this study (Table 1).

A) Longitudinal serum samples from horses experimentally infected with WNV lineage 1 (cohort A1, n=23), WNV lineage 2 (A2, n=3), JEV (A3, n=5), TBEV (A4, n=1) or USUV (A5, n=1). Infection of the horses was confirmed by virus isolation, RT-PCR and/or by plaque reduction neutralisation test (PRNT).
B) Serum samples from horses naturally infected with WNV lineage 1 from the USA (B1, N=27), WNV lineage 2 from Italy (B2, n=25) or TBEV from Austria (B3, n=60). Horses naturally infected with WNV lineage 1 (B1) were selected based on neurological signs and detection of anti-WNV IgM by capture ELISA. Serum samples from horses naturally infected with lineage 2 WNV were collected from clinically or asymptomatic animals during the Italian outbreaks that occurred in the period 2013-2014 and confirmed by IgG/IgM ELISAs and virus neutralisation test (VNT) (B2). Sera from horses with natural TBEV infection were obtained from an Austrian flavivirus population serosurvey (B3), confirmed by VNT.[22]
C) Serum samples from Dutch horses vaccinated for WNV (C1). WNV-vaccinated horses received an intramuscular injection with a licensed European WNV vaccine (inactivated whole virus; Duvaxyn WNV®, Fort Dodge) on day 0 and 21 days post primary vaccination (dpv). Serum samples from the vaccinated horses were obtained on different DPV.[23]
D) Serum samples from non-infected horses from the same region in Italy (D2, n= 50) and Austria (D3, n=12) as baseline groups for cohorts B2 and B3. As baseline group for cohort B1, pre-inoculation serum of horses in experimental WNV infections from the USA laboratory was used, as these horses were acquired on a local market. All sera were pre-screened by PRNT for the absence of WNV or TBEV antibodies.
E) Serum samples collected in 2005 from 138 horses (E1) across the Netherlands (presumed non-endemic to flavivirus circulation). WNV-free status was determined by ELISA and PRNT.[23]

We were not able to acquire sera from SLEV infected horses.

Antigen production

Due to its high immunogenic properties and virus specific epitopes [24, 25], NS1 can be used as an alternative to protein E in serological diagnostics to enable differentiation between serologically cross-reactive flavivirus infections.[26-28] Custom NS1 protein of JEV [genbank: NC_001437.1], WNV lineage 1 [genbank: EU081844.1], SLEV [genbank:ACB58159.1], TBEV [genbank:AAA86870.1] and USUV [genbank: NC_006551.1] were produced in human embryonic kidney 293 cells (HEK293-cells) to ensure proper folding, glycosylation and dimerization. Production and protein quality were confirmed by Western Blot (Immune

Technology Inc., New York, NY, USA). A 6xHis-tag used for purification by HPLC and a V5-epitope for quality control were added to the C-terminus of the NS1 proteins as previously described.[21]

Protein microarray slide preparation

Proteins were diluted in protein array buffer (Whatman, Maidstone, Kent, UK) containing protease inhibitor (BioVision, Mountain View, CA, USA) and spotted in triplicate for internal control on 16-pad nitro-cellulose coated glass slides (Maine manufacturing, GVS Group, Italy) using a non-contact protein array spotter (sciFLEXARRAYER SX spotter, Scienion, Germany). The optimal protein concentration for each antigen was determined by checkerboard titration as previously described.[20] After spotting, slides were dried and stored at room temperature in a climate controlled area with 0-10% humidity.

Protein microarray antibody detection

For antibody detection in horse serum samples, dried slides were treated with blocking buffer to reduce aspecific binding, serum was added in a 1:20 dilution and washed as previously described.[20] After washing, an anti-equine Fc-fragment specific IgG conjugated with an Alexa Flour-647 fluorescent dye (Jackson Immuno Research, West Grove, PA, USA) was added and incubated for 1 h at 37 °C. In the case of IgM detection an unlabeled conjugate of Fc5µ-fragment specific IgM (MyBioSource Inc., San Diego, CA, USA) was added followed by a tertiary antibody with label (Jackson Immuno Research, West Grove, PA, USA) and incubated for 1 h at 37 °C. After washing, slides were treated with sterile water for 2 min to remove excess salt and dried and scanned using the TECAN PowerScanner version 1.2 microarray scanner. The median spot fluorescence intensity (FI) per triple protein spot for each serum sample was determined by ScanArray Express 4.0.0.0001 supporting program (PerkinElmer, Waltham, MA, USA). Fluorescent signals have a minimum intensity of 0 fluorescent units and can reach a fixed maximum intensity of 65,535 fluorescent units when saturated. Data were imported and processed in the statistical software ‘R’ version 3.0.1 for further analysis.

Protein microarray data analysis

A script was written in R using the additional package ‘drc’ version 2.3-7 to convert the median spot fluorescent intensity (FI) into dose-response curves per protein for each serum sample.[20] Antibody titres were defined as the serum concentration that provokes a response half way on the dose-response curve between the minimum and maximum signal (EC50). Paired and unpaired Mann-Whitney tests were used to compare medians. Optimal titre cut-offs were achieved by selecting the highest possible combination of sensitivity and specificity through ROC optimal curve calculations performed in GraphPad Prism.[29]

Quality control slides

Anti-V5-epitope monoclonal antibodies (Invitrogen, Thermo Fisher Scientific, Rockford, MA, USA) were used to ensure the quantity of the protein spots of each batch. Optimum protein concentrations were defined as those at which maximum fluorescent signal and overlapping s-curves were achieved for anti-V5-epitope monoclonal antibodies and were found to be around 0.5 mg/ml for all proteins. To minimize batch-to-batch variations all printed batches were normalized by testing one slide from each batch with a serial dilution of anti-V5 monoclonal antibodies and calculate the corresponding titre as described above to one

Table 1. Horse sera

Virus or vaccine	Strain	Group number	infection mode	# serum samples	# animals	DPI/DPV range	Confirmatory test	Country of origin	Source
WNV lineage 1	NY99	A1	Experimental	62	22	0-21	VI, PCR, PRNT	USA	Richard Bowen, Colorado State University, USA
	NY99	B1	Natural	27	27	Unknown	ELISA IgM	USA	Vickers, University of Kentucky, USA
	IS98-ST1	A1	Experimental	4	1	8-35	VI, PCR, PRNT	France	ANSES - Laboratory for Animal Health, Maisons-Alfort, FR
WNV lineage 2		A2	Experimental	48	2	0-70	VI, PCR, PRNT	South-Africa	Alan Guthrie University of Pretoria, Equine Research Centre South-Africa
	Aus08	A2	Experimental	4	1	8-35	VI, PCR, PRNT	France	ANSES - Laboratory for Animal Health, Maisons-Alfort, FR
		B2	Natural	30	30	Unknown	PRNT	Italy	Davide Lelli, Istituto Zooprofilattico Sperimentale della Lombardia ed Emilia Romagna
JEV	Genotype I	A3	Experimental	12	4	0-14	VI, PCR, PRNT	USA	Bowen, Colorado State University, USA
	Nakayama	A3	Experimental	4	1	8-35	VI, PCR, PRNT	France	ANSES - Laboratory for Animal Health, Maisons-Alfort, FR
USUV	SAAR-1176-South Africa	A5	Experimental	4	1	8-35	VI, PCR, PRNT	France	ANSES - Laboratory for Animal Health, Maisons-Alfort, FR
TBEV	Hypr	A4	Experimental	4	1	8-35	VI, PCR, PRNT	France	ANSES - Laboratory for Animal Health, Maisons-Alfort, FR
TBEV	Unknown	B3	Natural	61	61	Unknown	PRNT	Austria	Norbert Nowotny, Veterinärmedizinische Universität Wien (Vetmeduni Vienna)

WNV vaccine	Duvaxyn WNV®	C1	-	35	5	0-42	ELISA, PRNT	Netherlands	Faculty of Veterinary Medicine, Utrecht University, NL[21]
Baseline group Austria	-	D3	-	12	12	-	TBEV PRNT	Austria	Norbert Nowotny, Veterinärmedizinische Universität Wien (Vetmeduni Vienna) [22]
Baseline group Italy	-	D2	-	50	50	-	WNV PRNT	Italy	Davide Lelli, Istituto Zooprofilattico Sperimentale della Lombardia ed Emilia Romagna
Baseline group Netherlands	-	E1	-	138	138	-	WNV ELISA + PRNT	Netherlands	Van Maanen, GD Deventer, NL[23]

preselected protein. The titre to this specific protein in all following batches printed was calculated in the same manner. The difference measured between the two batches was used as a correction factor for the whole slide. The ANSES (Laboratory for Animal Health, Maisons-Alfort, FR) serological reference sample (serum from a horse infected 35 days prior with WNV lineage 1) was included in each experiment to correct for day-to-day variability.

Results

Experimentally infected horses

The horses that were experimentally infected with one flavivirus showed seroconversion. Although there was some cross-reactivity, we could clearly distinguish between the specific flavivirus antibodies. To analyse homologous reactivity, we tested serum samples serially collected from horses that were experimentally infected with WNV lineage 1, WNV lineage 2, JEV, USUV or TBEV for reactivity with homologous NS1 as a first step in the validation process (Fig.1&2). In serum samples taken at 0 and 7 days post infection (dpi) the antigenic IgG and IgM reactivity to the homologous NS1 was low and comparable to each other (P>0.05) for WNV and JEV infected horses. In serum samples obtained at 14 and 21 dpi, the IgG and IgM reactivity to homologous NS1 was higher compared to 0 dpi (P<0.01). Serum samples obtained on 21 dpi for WNV and 14 dpi for JEV consistently showed a high antigenic IgG and IgM reactivity to homologous NS1. Based on ROC-calculation an IgG and IgM specificity of 100%, and a sensitivity of 95% for WNV and 100% for JEV was achieved with a cut-off titre of 1:20 at day 14 (JEV) and day 21 (WNV) post infection.

One horse showed higher IgG reactivity to WNV NS1 before infection in comparison to other horse serum samples, but showed seroconversion to IgM and a fourfold increase in IgG to WNV NS1 in follow-up samples (Fig.1). Sensitivity and specificity using samples of experimental infections could not be determined for TBEV and USUV as only one positive serum sample was available.

Flaviviruses are known to have higher cross-reactivity between IgG antibodies than between IgM antibodies [30]. To analyse the potential cross-reactivity of the flavivirus NS1 IgG antibodies to heterologous NS1, we calculated antibody titres for each serum sample for the cohorts of experimentally infected horses. In serum samples obtained on 0 and 7 DPI, the median antibody titres to all heterologous and homologous NS1 antigens were not significantly different (P>0.05). In serum samples obtained on 21 DPI, the median antibody titres to homologous NS1 were significantly higher compared to the non-corresponding antigens (P<0.01). (Fig. 2) This shows that on 0 and 7 DPI, insufficient antibodies were detectable to differentiate between infected and non-infected horses, while at 21 DPI sufficient antibodies were detectable.

To determine the usability of the NS1 microarray for diagnosing and distinguishing flavivirus infections in horses, and for public health purposes within a surveillance setting, we analysed the reactivity to NS1 in a flavivirus negative population. A total of 138 sera were collected from horses across the Netherlands in 2005 and used as a panel representing horses from a presumed JEV, USUV, TBEV and WNV non-endemic region. As WNV and TBEV were circulating in neighbouring countries (Germany and Belgium) during 2005 the sera were screened for WNV and TBEV using ELISA followed by PRNT. All sera were additionally tested by microarray for antibody titres to flavivirus NS1. Sera from nine horses had titre results that were defined as non-specific because they did not produce s-curves in the serum titration and showed non-logarithmic reactivity to all NS1 antigens including non-equine flaviviruses. The frequency of false-positive titres of >20 for IgG was low, namely 6/129 (5%)

for WNV, 7/129 (5%) for JEV, and 0/129 (0%) for USUV. False-positive titres for TBEV were detected in slightly more animals, namely 13/129 (10%).

WNV vaccination in horses

An inactivated whole-virus JEV-vaccine available for use in horses, allows the capability of differentiating between horses that were previous vaccinated and JEV-infected horses. This principle, also called Differentiating Infection from Vaccinated Animals (DIVA), is important for diagnostic and public health reasons. An inactivated whole-virus WNV vaccine is also currently used in the Netherlands. To determine the possibility to differentiate between NS1 antibody titres of WNV inactivated whole virus vaccinated and WNV infected horses, we measured multiple flavivirus (WNV, JEV, SLEV and USUV) antibody titres in serum samples obtained from WNV-vaccinated horses (C1) and WNV infected horses (A1+A2) (Fig.3). In serum samples obtained on 28 and 42 DPV (A1+A2), the mean antibody titre to WNV NS1 showed no statistical difference to titres of vaccinated horses (C1) (P=0.74).

The antibody titres in the serum samples of WNV-vaccinated horses (C1) only started increasing at 20 DPV, while the antibody titres in the serum samples of WNV-infected horses (A1+A2) started increasing at 14 DPI (Fig. 1). The antibody response of WNV-vaccinated horses was delayed compared to WNV infected horses and also showed a larger variation in degree of response (Fig. 3).

Natural infections of horses.

We field-tested the microarray for use in three different field settings: 1) WNV in horses with clinical WNV symptoms (B1), 2) WNV in infected horses with neurological symptoms and asymptomatic horses from the same area during WNV outbreaks (B2), and 3) TBEV in a horse population screening program (B3).

Overall, the median antigenic reactivity to WNV NS1 in all three field-settings was significantly higher in serum samples obtained from horses naturally infected with WNV compared to their negative controls (P<0.01) (Fig.4). In the field infections the microarray identified 93% (25/27) of the positive serum samples for WNV lineage 1 and 100% (25/25) WNV lineage 2. Due to limited resources, we could only able to validate IgM antibody response for WNV lineage 1 horses. At an IgM titre of 20, 80% (24/30) of the IgM positive WNV lineage 1 serum samples were identified (Fig.4). The median antigenic reactivity in the Austrian horses to TBEV NS1 (B3) was significantly higher in serum samples obtained from horses naturally infected with TBEV compared to negative controls (P<0.01). 87% (52/60) of the horses were diagnosed as TBEV IgG positive at a titre of 20 (Fig.4). One TBEV IgG positive horse and three TBEV IgG negative horses showed a linear signal and reactivity to all NS1 antigens and were thus classified as non-specific.

To assess the reactivity to multiple flavivirus antigens in serum samples obtained from horses with confirmed WNV (B1, B2) and TBEV (B3) field infections, we also measured antibody titres to WNV, JEV, TBEV and USUV NS1 (Fig.4). The median antibody titres to all homologous NS1 antigens were significantly higher in serum samples obtained from horses naturally infected with WNV compared to heterologous antigens (P<0.05). The median antibody titres to TBEV NS1 were higher than to the heterologous antigens in the respective TBEV infected horse population with one exception: one horse naturally infected with TBEV also had an equally high titre to WNV.

Figure 1: IgG and IgM response to multiple flavivirus NS1 antigens in sera collected from horses experimentally infected with WNV or JEV

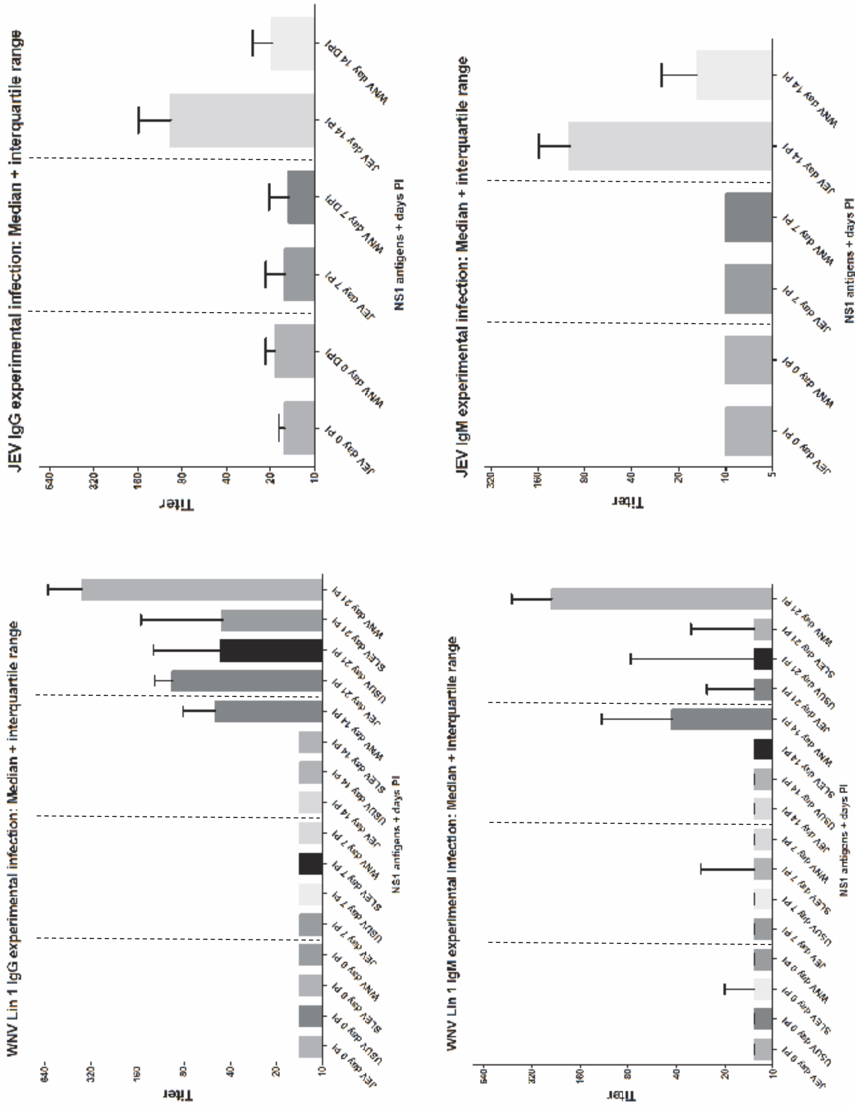


Figure 2: IgG response in sera collected from a single horse experimentally infected with USUV or TBEV 8 to 58 days post infection (DPI)

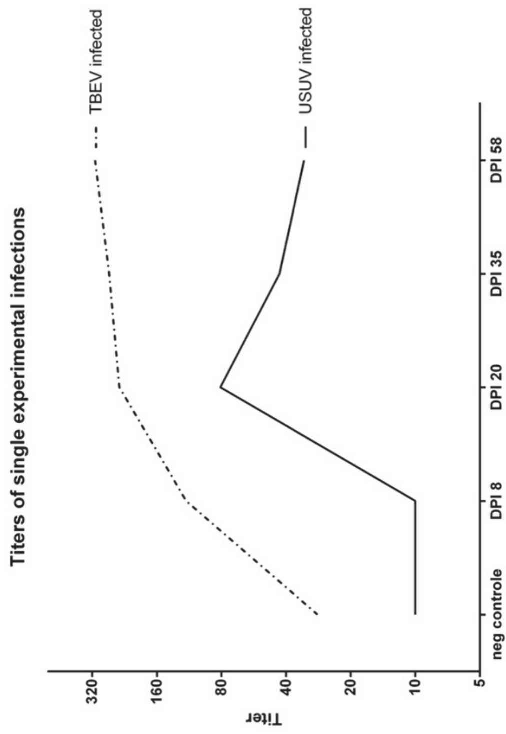


Figure 3: Mean (A) IgG and (B) IgM titers with SD of vaccinated horses with whole inactivated WNV vaccine days post vaccination (DPV).

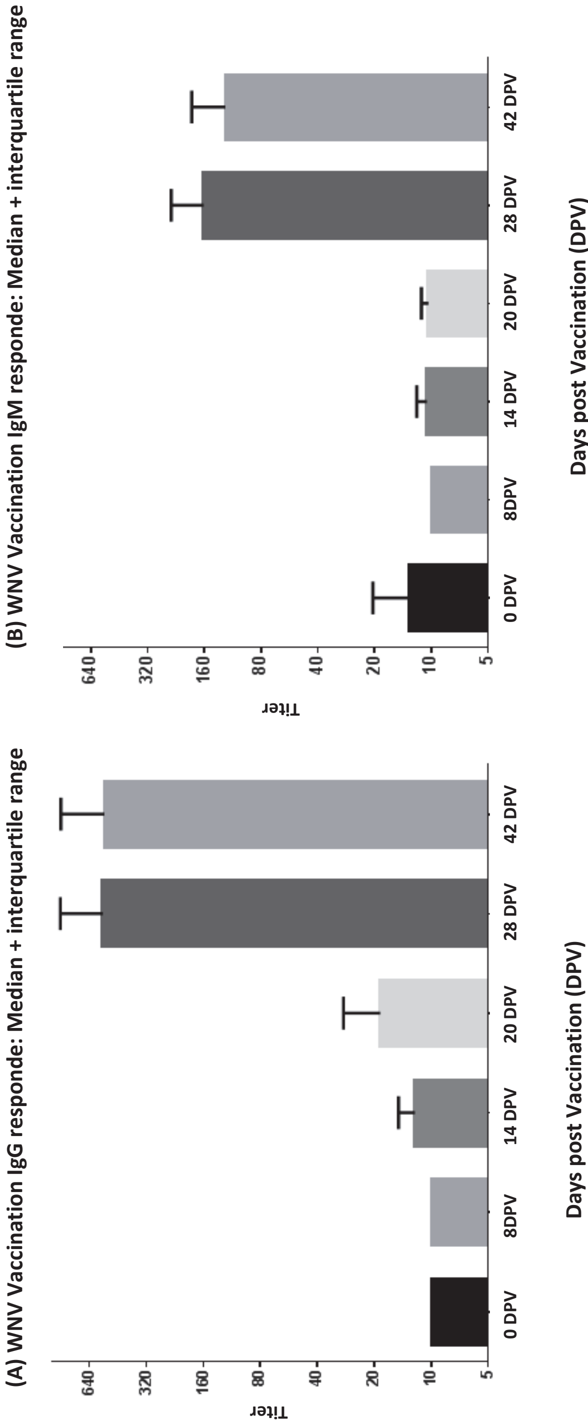
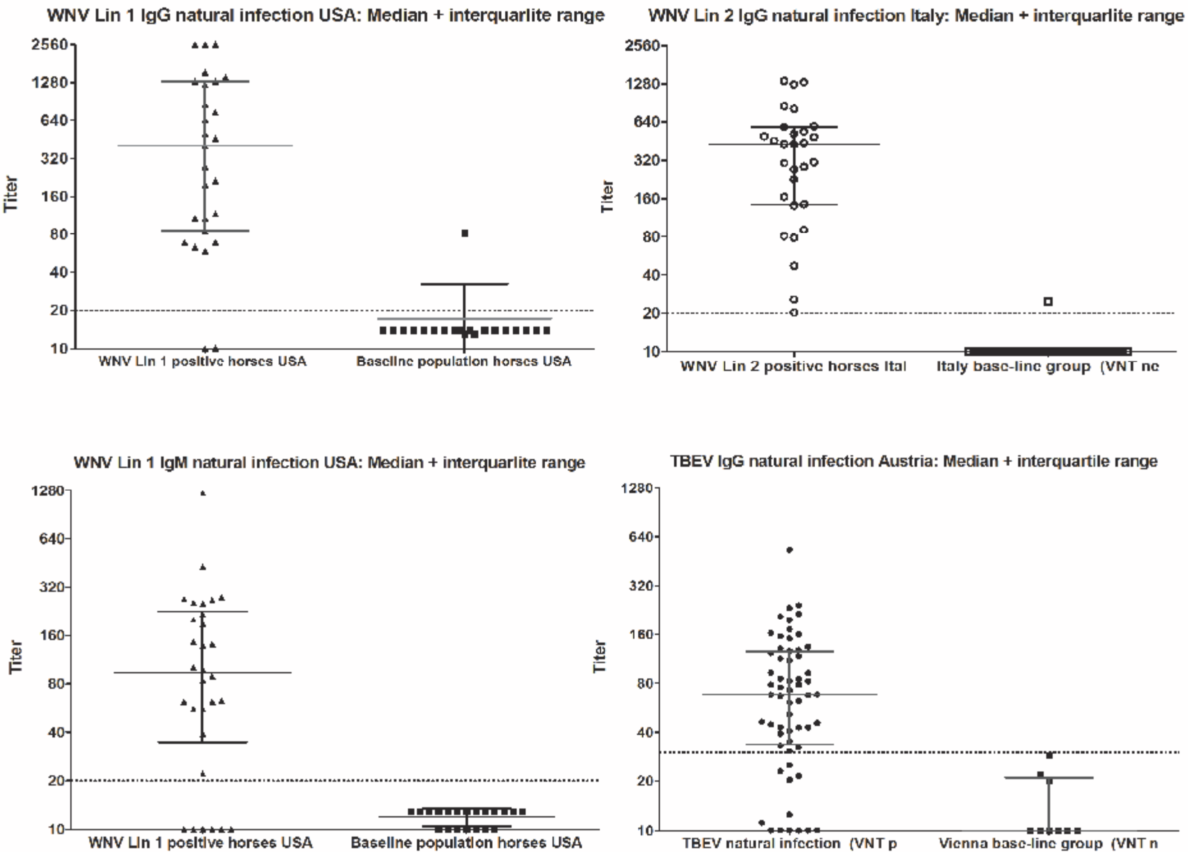


Figure 4: Use of the serological microarray in field setting for detection of exposure to WNV lin1 (USA), WNV lin2 (Italy) and TBEV (Austria) in comparison to VNT/ELISA



Discussion

We describe the development and implementation of a flavivirus multiplex serological protein microarray for the differential diagnosis of JEV, WNV, TBEV and USUV infections in horses. The results in experimentally, naturally and non-infected horses indicate the ability of the array to detect specific antibody responses. Stronger results could have been achieved if a larger number of well-defined sera of the less common flavivirus infections in horses, such as SLEV and USUV, had been available. The microarray could be a future tool for multiplex syndromic diagnostics and used for early detection of emerging vector-borne flaviviruses.

Sensitivity for WNV IgM and/or IgG

The array is less sensitive compared to the prM-E ELISA, possibly due to a later or lower antibody response to NS1 antigens, as previously established on Dengue virus in humans.[31] In a previous study, antibody titres to NS1 were lower compared to those against the whole envelope protein. This resulted in titres closer to the test cut-off. NS1-antibody detection methods could therefore potentially be more sensitive to variations in time post disease onset or natural horse-to-horse variations.

The time of infection relative to blood sampling may also have played a role in the sensitivity of the microarray in horses. In some cases, blood samples were likely taken too early post infection to have developed detectable IgG antibodies to NS1.

Differences in the antibody responses of these horses have also been observed by IgM-capture ELISA, and may depend on host genetic differences as well.[32] The currently measured low IgM sensitivity means that the use of the array for diagnostics in areas with high vaccine coverage or infection prevalence is limited as a first line test. IgM capture ELISA in humans and horses with neurological signs is the preferred diagnostic method, because detectable antibodies are already present at the onset of neurological signs. The lower IgM sensitivity of the microarray compared to the IgM capture ELISA may results in more false negative diagnoses. This reduces its usability for diagnostics and syndromic surveillance (neurological symptoms).

Increasing the IgM sensitivity might be achieved by combining NS1 antigens with envelope antigens and monoclonal antibodies in the future.[33] Because the specificity of the multiplex microarray is high and because differentiation is possible despite cross-reactivity, the test could be useful in serial or parallel testing schemes. Since the microarray shows good potential for IgG detection in low vaccine coverage areas, it should be particular useful for diagnostics and epidemiological studies in such areas.

Sensitivity for TBEV IgG

A high sensitivity for WNV IgG was achieved but the sensitivity for TBEV IgG could not be established to a comparable degree. We calculated the sensitivity for TBEV IgG on naturally TBEV-infected horses because only one experimentally TBEV infected horse serum was available. The achieved sensitivity for TBEV IgG in the field samples was not as high as for WNV and JEV and overlapped with the negative controls. This made determining a cut-off between positive and negative titres less clear. The lower titres may be due to the difference in population characteristics. The TBEV population used in this investigation were identified during cross-sectional surveillance programs. These horses had an unknown infection date and were not selected based on clinical symptoms. This may have resulted in more horses

having lower antibody responses due to infections months or years in the past or a faster decline of TBEV antibodies post infection compared to WNV.[22, 34]

Broad non-specific response in horses

The microarray showed the potential to achieve high sensitivity and specificity even though in a small number of field-infected horses non-specific responses were recorded. One horse experimentally infected with WNV showed by microarray a WNV titre (>20) before infection, although this serum was WNV negative by PRNT. Antibodies against WNV were confirmed after experimental infection, indicating that the previously measured titre was possibly a non-specific response or represents a broadly cross-reactive response. Such an antigenic response could also be seen in a small number of flavivirus antibody negative horses both from endemic and non-endemic regions. Nine WNV antibody-negative horses from the Netherlands showed reactivity to NS1 antigens that did not produce the expected s-curve titre during titration, but showed a linear signal reduction. Also these horses showed antibody reactivity to all NS1 antigens on the microarray at equal levels. Therefore it was not possible to calculate any titre even though a measurable signal was detected.

This broad non-specific response in flavivirus antibody-negative samples was not seen in arrays developed for other mammals.[20, 21, 35, 36] A possible explanation is that equine blood has a higher viscosity compared to other mammals due to differences in erythrocyte and plasma protein concentrations and composition.[37] This viscosity even increases when horses are highly active and stressed.[38] The microarray is based on nitrocellulose fibre membrane that catches proteins in its fiberous structure. Possibly, equine blood characteristics combined with the nitrocellulose platform may result in broad non-specific signals in comparison to other mammals.

Additionally, the antibody-negative group consisted of horse serum samples submitted to the Animal Health Services, Deventer, for a diverse range of diagnostic investigations, and the health status of these horses was not specified. Therefore, unknown factors could have interfered with the microarray in case of these nine horses. Epitope-mapping on the flavivirus NS1 exhibited cross-reactive epitopes with human endothelial cells.[24] In auto-immune diseases this might result in false-positive test results due to cross-reactivity.

Cross-reactivity

Cross-reactivity was measured between heterologous NS1-antigens, but the level of cross-reactivity was minimal and differentiation could be made. Low but detectable titres to heterologous NS1 antigens were measured, which is most likely a result of antigenic cross-reactivity.[39, 40] The fact that cross-reactive antibodies were measured to multiple antigens underlines the need for multiplex testing to identify the actual infection when using serology.

More cross-reactive antibodies were observed in naturally infected horses than in the experimentally infected ones (Fig.4). In most serum samples, differentiation between related flavivirus infections was possible because the titres against the corresponding NS1 antigen were higher compared to the non-corresponding NS1 antigens. However, some serum samples from naturally infected horses showed comparable or higher antibody titres to non-corresponding antigens. This might be influenced by the above-mentioned viscosity of horse sera, and flavivirus antibodies are known to be highly cross-reactive.[41, 42] These higher heterogeneous IgG titres may have been induced by previous exposure to a flavivirus

infection.[41] This was only observed for IgG, showing that IgG antibodies were more cross-reacting than IgM.

Vaccination
Previous studies have shown the possibility to differentiate between JEV infection and vaccination in human and horse populations.[27, 43] In contrast to the JEV vaccine, the inactivated whole WNV vaccine induces anti-NS1 antibodies after multiple injections.[44, 45] An NS1-based protein microarray can therefore not be used to differentiate infection from vaccination when using inactivated whole-virus WNV vaccines as shown in this study.
Additionally interesting, is it that all five horses showed a clear IgM reactivity >20 days post vaccination, which corresponds to the second (booster) WNV vaccination on day 21. In a previous study, were these serum samples originated from, only limited IgM reactivity was measured towards the PrM-E WNV antigen.[23] This limited reactivity was only seen in about 50% of the horses within first two weeks after the first vaccination and within one week of the second vaccination. IgM reactivity towards an inactivated vaccine is considered to be minimal or absent, however previous studies have shown conflicting results in this area.[23] Our results further support the claim that inactivated vaccines can induce IgM antibody production, at least towards NS1. However, the antibody reactivity is only seen after the second vaccination. NS1 antibodies are none-neutralizing, however, it is well known that they are strongly immunogenic and there possible importance in development of protective immunity has been indicated.[21] Our current results might further support the idea of a possible role for NS1 antibodies in developing protective immunity towards WNV. Further research into why a delayed NS1 reactivity is seen after vaccination and its possible role in development of immunity is needed in this area as it could have implications for future vaccine development.
A number of other vaccines are available in the European vaccine market that can support DIVA testing, for example recombinant canarypox vaccine (Protequ WNV, Merial) and the Yellow Fever virus chimeric vaccine Equilis West Nile (MSD Animal health).[46] The recombinant vaccine contains canarypox virus with WNV PrM and E viral antigens, and therefore does not induce flavivirus NS1 antibody production. Using diagnostics based on the NS1 antibody response can thus provide a sensitive method to identify exposure to WNV in areas where such vaccines are used. The chimeric YF-WNV vaccine may offer similar possibilities in horses, but may also generate false positive results through cross-reactivity with YFV NS1 antibodies. However, a study on the use of the array in humans, previously published by our group, has shown that this cross-reactivity is very limited.[21].
Additionally, due to the adaptable and multiplex setup of the microarray, YFV NS1-antigens can easily be added to the microarray to identify infected animals as well as vaccinated equids. Within Europe and North America YFV is currently not circulating, consequently, identifying antibodies to this virus in equids is very unlikely. Also, adding additional antigens like the envelope and PrM to the microarray could further strengthen its sensitivity and its DIVA capacitiy.

Conclusion
Multiplex serological protein microarray based on NS1 shows potential to discriminate flavivirus-infected from non-infected horses. Although serologic cross-reactivity does occur among antibodies to flavivirus NS1 antigens, differentiation between closely related flaviviruses is possible. We showed that the NS1-microarray can potentially be used for

diagnosing and distinguishing flavivirus infections in horses, and for public health purposes within a surveillance setting. The NS1-based protein microarray cannot be used to discriminate between flavivirus antibodies in horses in response to natural infections and antibodies induced by inactivated whole-virus WNV vaccines. However, new recombinant and chimeric vaccines have potential DIVA properties. Its easily adaptable multiplex setup makes the microarray specifically ideal for large-scale studies and can therefore be of great value as a multiplex diagnostic and surveillance tool in veterinary and public health.

Competing interests: None

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Retrospective study in travellers shows underdiagnosis of flavivirus infections, stressing the need for multiplexing

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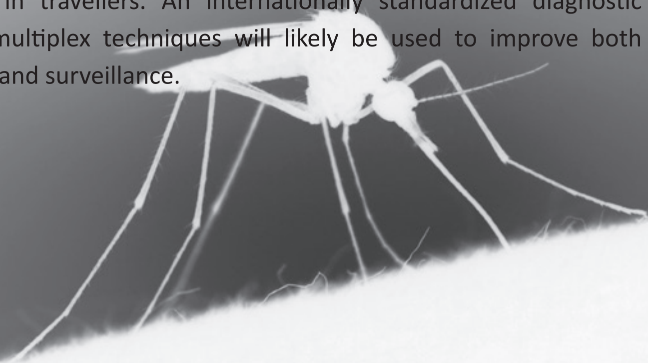
Abstract

Background: Routine evaluation of returning sick travellers is largely limited to the more well-known tropical diseases like malaria, rickettsia disease and dengue. We evaluated and quantified the additional patient and public health information that a standardized approach provides for diagnosis of flavivirus infections in travellers.

Method: We retrospectively tested patients with suspected arbovirus infections for dengue virus 1-4, West Nile virus, Japanese encephalitis virus, tick-borne encephalitis virus, Zika virus and yellow fever virus, depending on travel history. Retrospective serological diagnostics were performed using a protein microarray for multiplex serological diagnosis of cross-reactive flaviviruses. In addition, patients were tested by RT-PCR.

Results: Of the 436 patients previously tested through non-standardized routine diagnostics 52 (12%) had evidence of a flavivirus infection. Standardized flavivirus diagnostics, performed by PCR and microarray according to travel history, showed an increase of 58% to 82 patients that had evidence of a recent flavivirus infection. The missed diagnoses were DENV (84%), JEV (2%), WNV (2%), TBEV (5%), ZIKV (2%), and YFV (4%) infections.

Conclusion: Current, non-standardized diagnostic algorithms result in underdiagnosis of flavivirus infections in travellers. An internationally standardized diagnostic algorithm and use of multiplex techniques will likely be used to improve both travellers' health advice and surveillance.



Submitted

Introduction

Within the last decades, the world tourism industry has increased to over 1.1 billion international arrivals in 2014. Tourism to emerging economies is expected to increase from 45% in 2014 to 57% of the international arrivals in 2030.[1] The increase in international travel and trade has played a large role in the increase in exposure and geographical expansion of tropical diseases globally.[2, 3]

Data and information on tropical arthropod-borne (ARBO) flaviviruses is scarce. Diagnostic evaluation of returning sick travellers mostly is limited to the more well-known tropical diseases like malaria, rickettsiosis and dengue fever. Extending the diagnostic panel may improve patient care when it informs treatment decisions. In addition, evaluation of infectious disease syndromes in travellers can provide essential information on the activity of the diseases in the countries they visit where limited or no surveillance is available.[4, 5]

Diagnosis of flavivirus disease is challenging, due to the geographical overlap in occurrence of multiple medically important flaviviruses, the non-specific clinical presentation, and the cross-reactivity in serological assays with vaccine-induced antibodies. [4, 6-10]. Most importantly, however, the rapidly changing epidemiology of flaviviruses makes it difficult to keep up to date with the potential panels of arboviruses that travellers may have been exposed to. Extensive underdiagnosis of arboviral diseases outside the flavivirus genus that present comparable symptoms to dengue has been shown in the past.[10]

Within the flavivirus genus the potential for underdiagnosis may be further increased when false-positive tests stop further investigation. The recent outbreak of Zika virus in the Americas and in 2007 in French Polynesia is an example of the fast spread of flaviviruses with dengue-like symptoms that have previously remained obscure.[11, 12] Initially, Zika virus cases were misdiagnosed as dengue, as cross reactive IgM antibodies have been detected. The current outbreak suggests more severe complications than previously recognized although retrospective studies show that these complications may have been missed.[10, 13, 14]

As a potential solution for improving the information on vector-borne flaviviruses as well as for improving the diagnosis of travellers' diseases, we previously developed a syndrome-based decision support map to guide diagnostic requests for the most common arboviruses by presenting syndrome and by region. Here we evaluated and quantified the additional patient- and public health information that could be obtained by an algorithm based approach for diagnosis of flavivirus infections compared to current routine practices (physician-guided). We used a multiplex serological assay for flavivirus with a high differentiating capacity for expanding our diagnostic capacity.[15]

Method

Ethical Statement

This research was conducted in accordance with the Dutch law on medical research (WMO), article 1, and the study protocol was reviewed and approved by the Erasmus MC medical ethical committee (MEC- 2014-438). In compliance with Dutch Law and medical ethical guidelines, no personal identifiers were included. The selected patients were checked against an opt-out database to insure all participants consent to use of material for research.

Study subjects

Patients

Patients presenting for diagnostic testing at a hospital or health clinic in 2011 and 2012 because of a suspected arbovirus infection were included. Patient eligibility selection criteria were: recorded clinical symptoms corresponding to an arboviral infection (one or combination of undifferentiated fever, rash, arthralgia, neurological symptoms and/or haemorrhagic symptoms), travel history abroad to one of three regions (Africa, Asia or Europe), a current Dutch resident and the availability of sufficient serum for additional diagnostics. Serum samples were retrieved from biobanks at the Erasmus Medical Centre in Rotterdam and The National Institute for Public Health and the Environment in Bilthoven. Patients from a larger range in years, 2009-2013, with known onset of symptoms 0-7 days prior to sample collection, were selected for molecular testing as 2011-2012 provided insufficient available samples.

Patient categories

Patients were subdivided into groups based on A) travel destination (continent), B) symptoms at presentation and C) days post onset of symptoms, if available. Patients with samples taken 0 to 7 days post onset symptoms were tested by PCR. All other patients were tested by IgM and IgG serology composed of standard diagnostics supplemented by multiplex protein microarray serology. Patients were tested according to travel history to make relationship with clinical symptoms possible for later evaluation and identification of high-risk patients groups. (Figure 1)

Testing algorithms

Testing algorithms were based on a review of likelihood of infection for a given travel history, based on an exhaustive review.[16] Patients with travel history to Asia were tested for DENV1-4, WNV, JEV, ZIKV and TBEV. Patients with travel history to Africa were tested for DENV1-4, WNV, ZIKV and YFV. Patients with travel history to Europe were tested for DENV1-4, WNV and TBEV. (Figure 1)

Laboratory analysis

Polymerase Chain Reaction

Serum samples were screened for the presence of virus RNA by a real-time RT-PCR at the Erasmus MC based on previously published methods for DENV1-4 [17, 18], WNV [19], JEV [20], TBEV [21] and ZIKV [22]. RNA was extracted using MagnaPureLC (Roche Diagnostics, Almere, The Netherlands) and an internal positive control, Phocine Distemper Virus, was added to check proper extraction procedure. Eight µl extracted RNA was amplified in a 20µl final volume one-step RT-PCR using TaqMan® Fast Virus 1-Step Master Mix (Lifetechnologies, Nieuwerkerk a/d IJssel, The Netherlands). Amplification was performed in a LC480 (Roche Applied Science, Almere, The Netherlands). Additional negative and positive controls RNA for each virus with known concentrations were added to each 96 well-plate. Primers and probes used are shown in Table 1.

Protein Microarray

Custom-made NS1 proteins produced in human embryonic kidney 293 (HEK293) cells to ensure proper folding, glycosylation and dimerization were used (Immune Technology Inc., New York, NY, USA. Proteins expressed were DENV1 (genbank:FJ687432.1), DENV2

(genbank:FJ744720.1), DENV3, (genbank:FJ744738.1), DENV4 (genbank:EU854300.1), JEV (genbank:NC_001437.1), YFV (genbank:JN620362.1), ZIKV (genbank:AFD30972.1), TBEV (genbank:AAA86870.1) and WNV (genbank:EU081844.1).

The microarray slides were prepared as previously described.[7] In short, NS1 antigens at concentrations of around 2mg/ml were mixed with protein arraying buffer (Maine manufacturing, GVS Group, Italy) and spotted in triplicate as a within-test control per pad. Antigens were spotted onto a nitrocellulose pad coated glass slide (Maine manufacturing, GVS Group, Italy) using a non-contact protein array spotter (sciFLEXARRAYER SX spotter, Scienion, Germany).

Patient sera were tested on dried slides as previously described.[23] In short, slides were first incubated in Blotto blocking-buffer (Thermo Fisher Scientific, MA, USA). Serum was diluted in four four-fold dilution steps (1:20 to 1:1280) and added to the pads for incubation. An Fc-fragment specific IgG or Fc5μ-fragment specific IgM specific conjugate with a Cy5-fluorescent dye (Invitrogen, CA, USA) was used for antibody detection. A median fluorescence signal (measured at 647nm) for each of the triplet spots per antigen was determined by ScanArray Express 4.0.0.0001 supporting program (PerkinElmer, MA, USA). Results were imported in R for titre calculation as previously described.[7]

Micro Virus Neutralisation Assay

Vero E6 cells were plated on 96 well-plates for 24 hours in DMEM with 10%FCS, 2mM HEPES pen/strep/L-glut and 7,5% NaHCO3 until a monolayer has formed. Serum dilution were made in two-fold steps (1:8-1:1024), using the same media, except with 3% FCS. Per well 100xTCID50% of virus was added to the serum dilution and incubated at 37°C for one hour. After incubation the serum and virus mix were pipetted onto the Vero E6 monolayer and incubated at 37°C for one hour before added another 100ul of media. Plates were checked on days 3-5 under a light microscope for the presence of CPE.

Interpretation of laboratory diagnostics

Patients with PCR results of <Ct 38,5 were considered PCR positive. Patients were considered 'confirmed' when an acute stage serum sample was DENV NS1 antigen positive or PCR positive.[7] Patients with IgM seroconversion or ≥ 4-fold increase in IgG titre in paired serum samples taken with a minimum of two weeks apart were considered probable cases. Patients with both detectable IgM and IgG in a single serum sample were considered probable cases.

Patients testing only IgM positive above a laboratory predefined cut-off when only one serum sample was available were considered possible cases.[6] Patients testing positive only for IgG in a single sample were considered inconclusive and thus not included in the diagnostic outcome. Patients with paired serum samples with no increase in antibody levels were considered negative. Patients for whom no dates of onset were reported testing negative with a single serum sample were considered inconclusive. (Figure 2)

Statistical analysis

The analysis was performed in STATA.[24] Multi-variable logistic regression, chi-test and test of proportions was calculated with an alpha set at 0.05.

Figure 1: Flowchart of diagnostic algorithm used, based on travel history

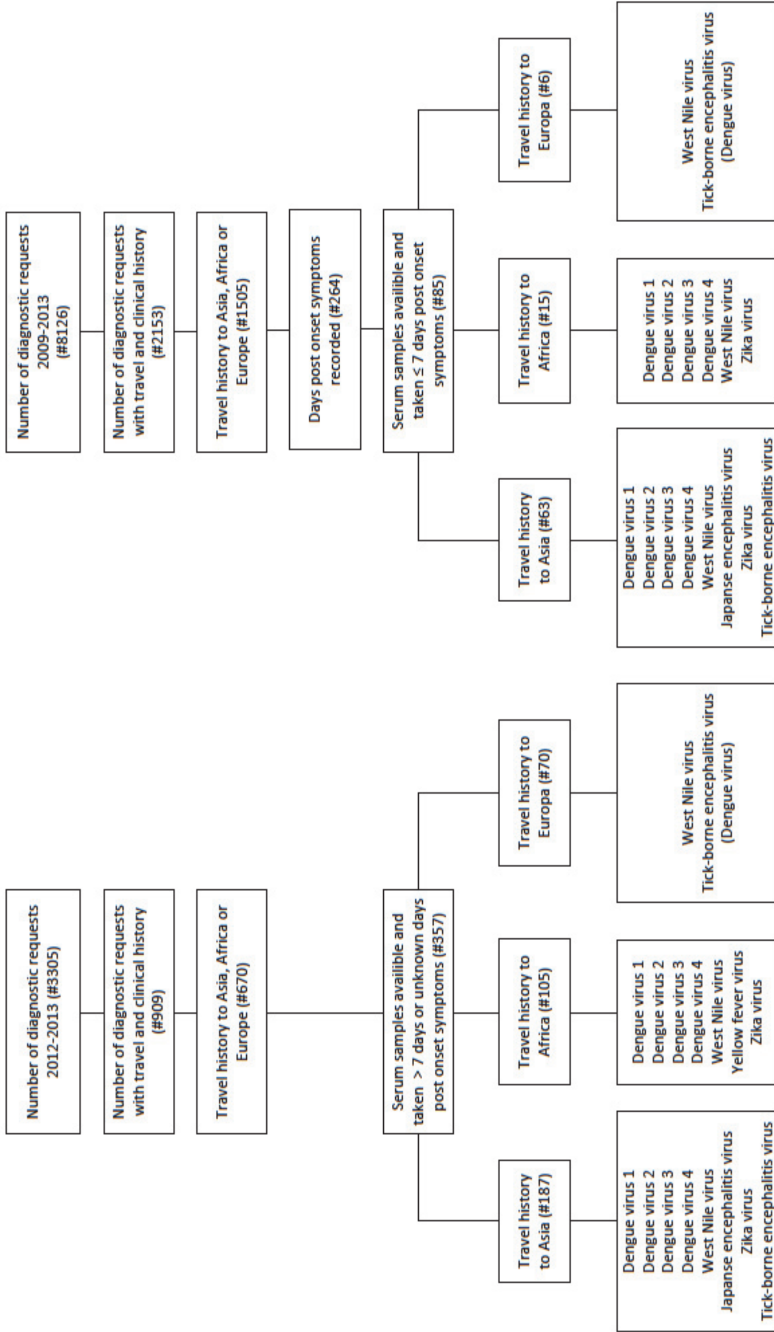


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Figure 2: Sample classification

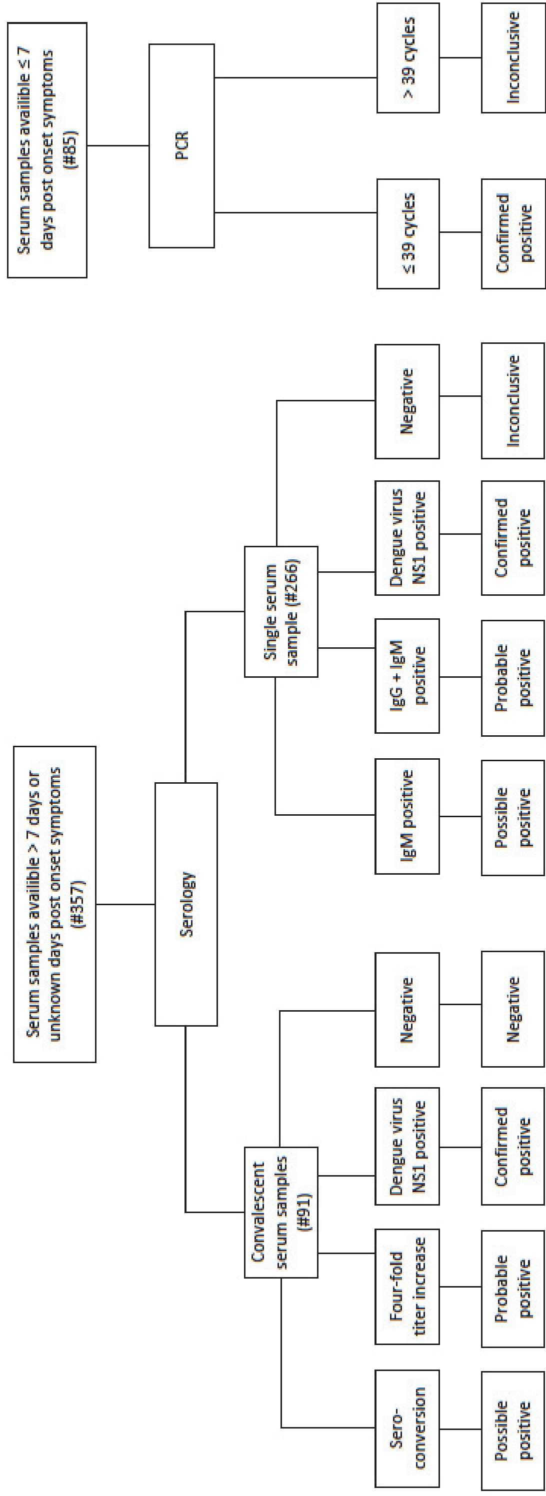

Table 1: PCR primers, probes and protocol used for molecular flavivirus diagnostics

Virus	Forward primer	Reverse primer	Probes: Fam…./…BHQ1	Reference
DENV1-4	DEN1: GACACCACACCCTTTGGACAA	DEN1: CACCTGGCTGTCACCTCCAT	DEN1: AGAGGGTGTTTAAAGAGAAAGTTGACACGCG	[17, 18]
	DEN2: CATGGCCCTKGTGGCG	DEN2: CCCCATCTYTTCAGTATCCCTG	DEN2: TCCTTCGTTTCCTAACAATCC	
	DEN3: GGGAAAACCGTCTATCAATA	DEN3: CGCCATAACCAATTTCATTGG	DEN3: CACAGTTGGCGAAGAGATTCTCAAGAGGA	
	DEN4: TGAAGAGATTCTCAACCGGAC	DEN4: AATCCCTGCTGTTGGTGGG	DEN4: TCATCACGTTTTTGCGAGTCCTTTCCA	
DENV all	GGATAGACCAGAGATCCTGCTGT	CATTCCATTTTCTGGCGTTC + CAATCCATCTTGCGGCGCTC (1:1)	CAGCATCATTCCAGGCACAG	
WNV	WNV1: CAGACCACGCTACGGCG WNV2: CCACCGGAAGTTGAGTAGACG	WNV1: CTAGGGCCGCGTGGG WNV2: TTTGGTCACCCAGTCCTCCT	WNV1: TCTGCGGAGAGTGCAGTCTGCGAT WNV2: TGCTGCTGCCTGCGGCTCAACCC	[19]
JEV	ATCTGGTGYGGYAGTCTCA	CGCGTAGATGTTCTCAGCCC	CGGAACGCGATCCAGGGCAA	[20]
TBEV	GGG CGG TTC TTG TTC TCC	ACA CAT CAC CTC CTT GTC AGA CT	WT TGA GCC ACC ATC ACC CAG ACA CA	[21]
ZIKV	CCGCTGCCCAACACAAG	CCACTAACGTTCTTTTGCAGACAT	AGCCTACCTTGACAAGCAGTCAGACACTCAA	[22]

Protocol: 5 min 50°C, 20 sec 95 °C, 45 cycli (3 sec 95°C, 30 sec 60°C)

Results

General dataset

All together, 436 patients were included in the study that met the selection criteria. The male/female ratio was 47/53. Patients had an average age of 38.9 years (SD of 16.6) on day of testing. Vaccination history was not provided with any of the patients. Most persons had travelled to Asia (56%) and presented with febrile symptoms (85%). Ten patients had travelled to more than one continent and were thus tested analysed based on multiple travel regions, but reported separately. Overview of reported travel history and clinical symptoms are shown in Table 2.

In total, 84 (19%) patients had a serum sample taken seven days or less post onset of symptoms and were tested by PCR. The mean number of days post onset of symptoms for these patients was 3.6 (SD=1.8). The remaining 357 patients were tested based on our extended algorithm. (Figure 1) For these, 17% (60/357) had travel dates provided. Sampling was done on a median of 21 days (ICR14-38.5) post onset of symptoms. In total, 91 patients (21%) had convalescent serum samples available.

Of the 436 patients previously tested through non-standardized routine diagnostics 52 (12%) had evidence of a flavivirus infection (Table 3). Applying the standardized flavivirus diagnostic algorithm increased the number of diagnoses by 58% (82 patients with possible, probable and confirmed (19%) of which six new patients were detected by PCR. The division between possible, probably and confirmed cases changed due the confirmation or identification of a different infection agent, of previous possible and probable cases through PCR and additional testing. This was significantly more than without standardized flavivirus diagnostics ($P<0.01$) (Table 3).

Travel region

The proportion of travellers testing positive differed significantly by region, but not when comparing results for non-standardized and algorithm-based testing (Table 5). The highest percentage of flavivirus positive patients had travelled to Asia. The results seen in Table 3 indicate a doubling of identification of the number of patients with evidence of the flavivirus infection in travellers to Southern Asia and Africa, however, no statistical significant differences could be measured due to the small number of patients per region. (Table 5)

Diagnostic yield

DENV infection was the most common diagnosis, and a substantial increase in diagnoses was found by routine molecular testing, despite the routine use of NS1 ELISA for DENV antigen detection. In the group of 84 patients tested by PCR, 32 had previously been tested for presence of DENV NS1 antigens. The DENV NS1-antigen assay had only detected 1 out of 5 PCR positive DENV patients in this group.

For the other flaviviruses, using an algorithm to inform diagnostic testing gave a more than 4 fold increase of diagnoses (2x JEV; 2x WNV; 2x ZIKV; 3x YFV; 4x TBEV) of which seven were classified as 'probable' and six as 'possible' infections. Two travellers returning from Indonesia and Ghana, respectively, had serological evidence of ZIKV virus based on IgG and IgM seroconversion. An additional patient from Thailand had a high IgG titres for ZIKV, however IgM results were showed no evidence for a recent infection. Three travellers from Africa (South-Africa, Uganda, Cameroon) had evidence of recent YFV infection or vaccination. No vaccination history was provided with these patients. One patient previously diagnosed with a WNV infection by IgM and IgG antibody titres was additionally found to

have a correspondingly high DENV4 IgM titre by microarray. All IgG titres were confirmed by mic-VNT with the exception of 1x JEV IgG and 1x ZIKV IgG due to insufficient serum.

DENV in Europe

As DENV is also occasionally detected present in the Southern part of Europe[25], 42 patients returning from Southern European countries or southern France were also tested by microarray for DENV1-4 antibodies to NS1. One patient showed evidence of a 'possible' DENV infection with an IgM titre after travel to Greece, but no follow-up samples. A second patient with travel history to Thailand two weeks before onset of symptoms had a five-day overlay in southern France before returning to the Netherlands. Nine days after return to the Netherlands the patient became sick and was tested NS1 and IgG & IgM DENV positive. No further evidence for missed flavivirus infections were measured in travellers returning from European countries.

Cross-reactivity

Due to the multiplex setup of the diagnostic microarray, possible cross-reactivity that occurred could be identified. The most commonly found cross-reactivity was seen between DENV IgG and YFV IgG. Three patients that had been defined as 'probable' DENV cases were found to have only DENV IgM titres on the microarray and accompanying high YFV IgG titres and were thus reclassified as 'possible' infections.

One of the two ZIKV positive patients found by protein microarray had been tested positive by DENV IgM assay in routine diagnostics, but could not be confirmed when the convalescent sample was tested. In the second ZIKV case a low IgG titre for DENV had been measured in the convalescent sample by routine diagnostics.

Table 2: Overview of travel history (UN definition) and clinical syndromes reported per patient

Travel history	Number of patients	Symptoms	Number of patients
Asia total	**248**	Febrile symptoms	369
Southeast Asia	189	Arthralgia/Arthritis	90
Southern Asia	39	Enteric symptoms	62
Eastern Asia	12	Rash	49
Western Asia	7	Respiratory symptoms	39
Africa total	**119**	Neurological symptoms	38
Eastern Africa	45	Haemorrhagic symptoms	16
Southern Africa	19		
Northern Africa	10		
Central Africa	9		
Western Africa	38		
Europe total	**75**		
Southern Europe	38		
Western Europe	22		
Eastern Europe	13		
Northern Europe	5		

Table 3: The number of *possible, probable* and *confirmed* patients before and after use of standard diagnostic algorithm for flaviviruses.

	Non-standardized diagnostics	Standardized diagnostics	*P-value*
Total	52/436 (12%)	82/436 (19%)	*P<0.01*
Possible	21/52	17/82	*P<0.01*
Probable	8/52	28/82	*P=0.01*
Confirmed	23/52	38/82	*P=0.8*

Demographics and symptoms

The DENV positive patients were the most commonly diagnosed flavivirus infections. Demographic data and symptoms at presentation were compared to those from patients without evidence of a recent DENV infection to see if these could be used to triage patients. Age, sex, reference laboratory, the number of samples provided or specialization of physician showed no relationship with the odds of a patient being classified as DENV positive. Before standardized diagnostics based on travel history was performed, only arthralgia showed a statistical negative relationship with predicting DENV positivity (OR=0.4, P=0.02). Travel to Asia but not to Africa had a significant positive predictive value for a patient to be DENV positive (OR=10.2, P=0.04). After diagnostics were performed according to travel history, haemorrhagic and febrile symptoms as well as travel to Asia or Africa had a predictive value for a patient having evidence of a DENV infection (Table 5).
DENV positive and negative patients did not differ regarding prevalence of enteric and neurological symptoms. Two patients with severe encephalitis and eleven patients with enteric symptoms were positive for DENV (Table 6).

Discussion

Here we evaluate and quantify the additional patient and public health information of a standardized approach for diagnosis of flavivirus infections compared to current routine practices that are often non-standardized. We found a significant (58%) increase in arbovirus infections diagnosed through the algorithm approach.

DENV infections accounted for 17% of patients with suspected arbovirus infections and 84% of all diagnoses. The number of DENV cases in travellers has been increasing extensively from around 6% in 1997-2006 to 12% of travellers returning with systematic diseases in 2000-2012, and is now listed as second most common cause of febrile illness in travellers, after malaria[26, 27]. Specific high peaks are seen in travellers returning from South America and Southeast Asia of up to 30-35% after 2010.[28, 29] Our findings are in line with observations by Prince et al (2009), and show that the sensitivity of the NS1-antigen capture ELISA is suboptimal. Additionally, these six patients did not have convalescent samples provided, which could have provided serological confirmation without the need of PCR. This need for convalescent samples is standard request for all arbovirus diagnostic tests but in practice not uphold causing problems for all serological diagnostics.

An important goal of the study was to investigate the possibility of missed infections with other flaviviruses, as these are often not requested in physician-guided diagnostic evaluations, as emphasized by the recent ZIKV outbreaks in French Polynesia and South America. Even with our small selection of travellers, 12% of flavivirus infections were

Table 4: Percentage and number of patients with evidence of flavivirus infections before and after standardized flavivirus diagnostics per region excluding travellers to more than one continent. (EU results not shown)

Continent	Region	Percentage positive with non-standardized *routine diagnostics* (Number of pos / number of tested)	Percentage positive after *standardized diagnostics* (Number of pos / number of tested)	
Asia (240)	**Total**	**18% (40/222)**	**24% (58/240)**	**P=0.1**
	West	0% 0/5	0% 0/5	ND
	South	9% (3/32)	18% (7/39)	P=0.28
	Southeast	21% (37/177)	26% (49/186)	P=0.22
	East	11% (1/9)	18% (2/11)	P=0.7
Africa (114)	**Total**	**8% (8/103)**	**15% (17/114)**	**P=0.1**
	North	11% (1/9)	11% (1/9)	ND
	South	0% (0/14)	17 3/18	P=0.1
	East	8% 3/38	14% 6/44	P=0.4
	West	9% 3/34	19% 7/36	P=0.2
	Central	11% (1/9)	11% (1/9)	ND
Africa-Asia		**P=0.02***	**P=0.05***	

* Asia total compared to Africa total
ND = Not Done

Table 5: Percentage and number of patients with evidence of flavivirus infections before and after standardized flavivirus diagnostics by serology (left columns) and PCR (right columns).

	Serology		Molecular	
	With non-standardized routine diagnostics % (Sero positive / Sero tested)	After standardized diagnostics % (Sero positive / Sero tested)	With non-standardized routine diagnostics (PCR pos / PCR tested)	After standardized diagnostics % (PCR positive / PCR Tested)
DENV	15% (39/267)	16% (46/289)	0% (0/3)	21% (16/78)
TBEV	6% (2**/32)	2% (4/255)	0% (0/2)	0% (0//69)
YFV	0% (0/1)	3% (3/105)	ND	ND
WNV	2% (1/54)	1% (2/357)	0% (0/2)	0% (0/84)
JEV	0% (0/12)	1% (2/187)	0% (0/1)	0% (0/63)
ZIKV	ND	1% (2/289)	ND	0% (0/78)
Total	13% (42/325)	17% (59*/357)	0% (0/8)	19% (16/84)

* One patient had evidence of WNV (IgG and IgM) and of DENV4 (IgM) infection. **The two TBEV positive patients were tested by microarray and PCR. ND = Not Done

Table 6: Multi-variable logistic regressions reporting odds ratios of possible factors related the odds that a patient is diagnosed as DENV positive versus DENV negative within the predefined patient group after standardized diagnostics. Also reporting the average age and percentages of the demographic and epidemiological characteristics of patients with and without evidence of a DENV infection after standardized diagnostics. Due to a strong correlation between febrile disease and the absence of neurological symptoms or arthralgia, both combinations have been calculated in a separate regression.

Regression 1 — **Standardized diagnostics**

LR chi2=20.42 p=0.04. Log likelihood = -183.18S

Independent factor	Odds ratio	CI 95%	P-value	DENV positive (#72)	CI 95%	DENV negative (#364)	CI 95%
Sex	1.0	0.6-1.7	0.97	47%	42-53%	48%	36-60%
Rash	0.9	0.4-2.2	0.90	11%	6-21%	11%	8-15%
Enteric symptoms	1.2	0.6-2.5	0.66	15%	9-26%	14%	11-18%
Reference lab	0.8	0.4-1.7	0.65	75%:25%	63-84%:16-37%	75%:25%	70-79%:21-30%
Multiple samples provided	1.2	0.7-2.4	0.45	24%	15-35%	20%	16-25%
Age	0.9	07-1.2	0.36	37.9 years	34.0-41.8	39.1 years	37.4-40.8
Respiratory symptoms	0.6	0.2-1.6	0.28	7%	3-16%	9%	7-13%
TM specialization*	1.6	0.9-3.0	0.13	45%	34-57%	38%	33-43%
Haemorrhagic symptoms	4.0	1.2-12.3	0.02**	8%	4-18%	3%	1-5%
Febrile symptoms	5.3	1.6-17.8	<0.01**	96%	87-99%	82%	78-86%

Regression 2 — **Standardized diagnostics**

LR chi =24.6 p=0.02. Log likelihood = -182.17

Independent factor	Odds ratio	CI 95%	P-value	DENV positive (#72)	CI 95%	DENV negative (#364)	CI 95%
Reference lab	1.0	0.5-2.0	0.93	75%:25%	63-84%:16-37%	75%:25%	70-79%:21-30%
Rash	1.0	0.4-2.2	0.90	11%	6-21%	11%	8-15%
Enteric symptoms	1.1	0.6-1.8	0.80	15%	9-26%	14%	11-18%
Sex	1.1	0.6-1.9	0.70	47%	42-53%	48%	36-60%
Age	0.9	0.7-1.2	0.43	37.9 years	34.0-41.8	39.1 years	37.4-40.8
Multiple samples provided	1.3	0.8-2.7	0.39	24%	15-35%	20%	16-25%
TM specialization*	1.5	0.8-2.7	0.24	45%	34-57%	38%	33-43%
Respiratory symptoms	0.5	0.2-1.4	0.20	7%	3-16%	9%	7-13%
Neurological symptoms	0.2	0.4-0.9	0.03**	3%	0.7-11%	10%	7-14%
Haemorrhagic symptoms	3.8	1.2-11.8	0.02**	8%	4-18%	3%	1-5%
Arthralgia-arthritis	0.3	0.14-0.8	0.01	10%	5-20%	23%	19-27%

* TM specialization = Travel medicine / Tropical medicine specialization. ** Not significant for non-standardized diagnostics

non-DENV cases, including two probable ZIKV cases. Prospective studies on arboviral causes for febrile disease in South American and African local populations have also indicated an exposure to an extensive selection of arboviral diseases, although the numbers are lower than for DENV.[30, 31]

In our patient group, a number of possible YFV infections or vaccinations were detected without registration of vaccination history although clinical symptoms were recorded. These patients were therefore clinically sick, however our microarray cannot distinguish between vaccination and infection. Import of YFV infections in returning travellers is frequently reported although vaccination is available.[32] Southern-Africa, however is not considered a YFV active area, so YFV infection in this patient is improbable. The closely related- and serologically cross-reactive Wesselsbron virus is known to circulate and cause clinical disease in South-Africa.[33, 34] We cannot rule out that this was the cause of the YFV serological. The extent to which YFV vaccinations, with live-attenuated virus, can influence flavivirus diagnostics is not properly recognized by treating physicians, as vaccination history rarely is provided with diagnostic requests in our setting.[6] More effort is needed in this area, as YFV is an important and preventable international disease.

We show that the quality of data that can be provided for surveillance and patient case definition increases after the application of systematic diagnostics. Before application of standardized diagnostics symptoms did not show and predictive relationship due to substantially smaller patient group compared to our previous publication.[35] Without changing the amount of data provided by physicians for patient diagnostics the data quality could be increased substantially just by applying structural diagnosis. The OR achieved in this study were comparable with previous published results on a larger dataset.[35] This shows that these predictive symptoms and travel combination, with further optimizations can surely also be used in travellers for identifying high risk patients.

An important question is whether the costs of expanding our diagnostic algorithm can be justified. Answering this requires an economic calculation that would have to be conducted taking all the financial costs and all the benefits to patient and societal health into account.[36] Only few arboviral vaccines are available and the added value of diagnosis would probably be limited to the impact on pre-travel advice for future travellers and prevention of complications and unneeded testing. On the other hand, improved testing provides an opportunity to understand better the epidemiology of flavivirus infection in this particular population of travelers. This could be used for evaluating the distribution of resources for research into treatment, prevention, and diagnostics.
Using current cost-benefit analysis methodologies, it is difficult to calculate the financial advantage of public health surveillance in preventing disease outbreaks. Evaluation and calculation of the impact of available intervention schemes in past outbreaks and the cost reduction in patient health will need further in-depth analysis for better prediction if there is potential added value and thus should be invested in surveillance of unknown diseases.[5]

Our study does have some limitations. The size of the study population was relatively small, as referring physicians rarely provide complete minimal data needed for inclusion. This reduces the power o the study for low prevalent infections. Also, there is a risk of bias, as physicians may be more careful with providing background data for specific patient groups. Finally, only patients seeking medical attention were included. Extrapolating exposure rates for surveillance purposes should thus be done with caution as this is a very specific high-risk group.

Conclusion

Nevertheless, our results show that combined serological and molecular techniques are essential for adequate DENV diagnosis of travellers as this increases the number of possible, probable and confirmed cases by 58%. Additionally, our study shows that travellers are exposed to other flaviviruses beyond DENV, but numbers are limited. Current, non-standardized diagnostics result in underdiagnosis of flavivirus infection in travellers. Internationally standardized diagnostic algorithms and use of multiplex techniques will likely improve both travellers' health and the surveillance of flavivirus activity and epidemiology.

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Conflicts of Interest: None to declare by all authors

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7

Summarizing discussion and conclusion

Summarizing discussion and conclusion

The goal of this thesis was to study how existing medical information and health structures can be used for arbovirus surveillance in the Netherlands. To achieve this, we combined and analysed all routine diagnostic data for Dutch travellers for suitability in arbovirus surveillance. We developed diagnostic algorithms to identify possible underdiagnosis of arbovirus infections in travellers. Based on these algorithms, we developed, validated, and applied a multiplex serological protein microarray in a retrospective study in travellers and outbreaks in equines to evaluate its added value in public health surveillance and patient care.

A critical look at travel medicine from the perspective of arbovirus surveillance
In **chapter 2** we investigated the possibility of using diagnostic results for individual patient care for surveillance of arboviruses, with travellers as sentinels for arbovirus activity in data-low countries, and assessment of arbovirus import risk through travellers. We showed that the data provided important surveillance information regarding dengue virus activity in countries visited by travellers and the risk of travellers returning in the viremic stage, although 95% of the data was serological and 90% missed convalescent sampling or molecular testing. The serological data, however, were suboptimal for specific disease surveillance due to limited confirmatory and molecular testing, and it relied on the large number of patients for use on a national level.

Data quality and compatibility in national Health Information Systems (HIS)
During the process of data extraction and integration for **chapter 2**, we discovered a number of challenges: the incompatibility of different Laboratory Information Management Systems (LIMS), lack of complete patient data and lack of standardized input and interpretation. The eventual amount of data that could be included based on availability of an essential minimal dataset for surveillance (was approximately 45%. In addition, nearly all patient data (99%) lacked information on vaccination history, impacting the specificity of the findings with a serologic diagnostic method.[1]
Timely availability of this data is essential not only for surveillance and early response but also for interpretation of patient laboratory results. Missing data has been detected in other secondary and tertiary healthcare facilities besides diagnostic laboratories.[2] For example, evaluations of request forms from haematology and radiology laboratories and hospital patient files have shown a lack of basic data, like birth dates and clinical information.[2-4] This lack is even more apparent in handwritten request forms or oral communication than in a digital Health Information System (HIS).[2, 4]
Missing basic information has resulted in misinterpretation of data, lack of supportive medical discussion by secondary and tertiary facilities that generates additional work and costs, and delayed results and conclusions.[2, 5] When HIS are used, they are often incompatible between different healthcare facilities, making data exchange between facilities, and sometimes even departments, difficult or impossible.
To identify the main data loss points in our study, we visualised the data flow from patient to arbovirus national surveillance through different information systems and categorized several points at which data could be lost (Figure 1). In our study, most data were exchanged via handwritten or printed forms requiring manual input into an HIS? Thus, data could have been lost because of lack of compliance with minimal data entry at any step in the chain.

A national compatible and linked laboratory information management system (LIMS) with digital standardized input (dropdown boxes) and compulsory information provision areas would resolve the issue of lack of, or limited, basic information. Current cost-benefit analyses of comparable HIS have shown that the savings in patient care resulting from complete documentation will equal the investment in the HIS within 3 to 13 years. However, it remains questionable if investment in such a system will be financially viable. Development of such a system can improve the quality and quantity of data, however, education of medical staff on the significance of proper and complete documentation for patient care and surveillance remains essential for compliance.[6]
Of additional concern regarding the linking of national LIMS are the ethical implications of patient privacy. Previous attempts in the Netherlands to implement data transfer between systems have resulted in resistance from the general population.[7] Although our surveillance method uses anonymous data, ethical consideration about the potential of a linked system to be easily expanded and hackable should be explored before further implementation.

A critical look at travel medicine from the perspective of arbovirus diagnostics
A national arbovirus working group was convened to inform stakeholders such as national health centres, clinicians, and physicians about the potential benefits of standardization of diagnostic information. Its findings revealed an unclear overview of current arbovirus diagnostic capacity in the Netherlands and the lack of standardized diagnostic algorithms for travellers for all arboviruses. Therefore, in **chapter 3** we developed diagnostic algorithms for cases of travellers with particular travel histories and particular syndromes that could be extended for surveillance.
Based on an extensive literature review of the risks of travellers contracting an arbovirus infection, we created a method to classify arboviruses by high, middle, low-to-no or unknown risk to travellers. Arboviruses with low-to-no risk to travellers were excluded, and the remainder were categorized by clinical syndrome, geographical distribution and potential serological cross-reactivity. This information was organized into charts with algorithms based on syndrome and travel history for fast referencing by clinicians. The charts were distributed via peer-reviewed English and Dutch medical journals and presentations at conferences, meetings and workshops, through medical and surveillance networks and directly to collaborating travel and tropical medicine clinics.
To establish the level to which arbovirus diagnostic requests in the Netherlands followed the advice in the literature, in **chapter 4** we retrospectively compared an optimal selection for arbovirus diagnostics with what was actually performed in the previous 5 years. Although many important differences between our optimal algorithm and the actual arbovirus diagnostics in the Netherlands were identified, the most important was the identification and classification based on the travel history and clinical symptoms of a large group (44%) of patients at risk of CHIKV infection for which no serological or molecular tests were performed.

Adapting public health surveillance systems to moving targets
In **chapter 3** we developed algorithms and charts to support the physician decision-making process and improve quality and quantity of data available for surveillance. The epidemiology of arboviruses, however, is constantly changing,[8] exemplified by the outbreak of chikungunya virus in the Indian Ocean and the Caribbean and Zika virus in

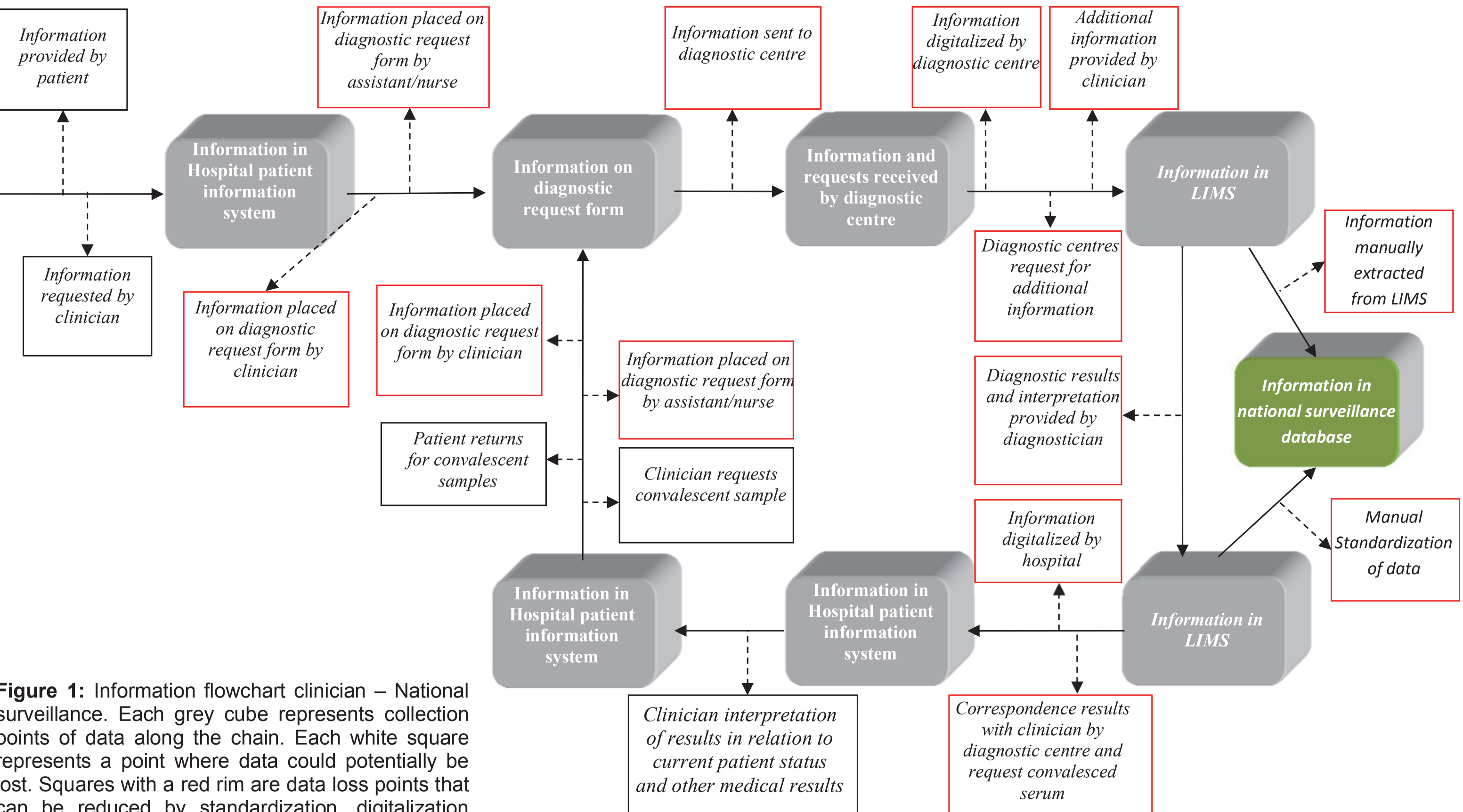


Figure 1: Information flowchart clinician – National surveillance. Each grey cube represents collection points of data along the chain. Each white square represents a point where data could potentially be lost. Squares with a red rim are data loss points that can be reduced by standardization, digitalization with compulsory sections and compatibility with a national system.

Polynesia and Brazil.[9, 10] How, then, can public health surveillance systems, like the one we propose, adapt quickly enough to changing public health needs?[11]
First, online (dropdown) diagnostic request forms could be easily updated by a public health agency on the basis of the latest surveillance data. A set of compulsory fields for physicians to complete when requesting a diagnostic test would lead to more comprehensive information for surveillance. Also, this set would reflect the latest surveillance information and could suggest which arboviruses (or other diseases) most likely fit the parameters in the diagnostic request form, helping the physician to request testing for the probable causes.[10] If the physician decides to request diagnostics for an arbovirus not suggested by the dropdown form, the system would notify the agencies responsible for surveillance. Despite previous implementation of compulsory fields and dropdown boxes in other countries,[10] willingness of clinicians to accept these kinds of interventions has not been established.
A third strategy for a system to adapt quickly to changing needs is syndromic surveillance [12]. Reliable and timely national surveillance is the foundation for good international surveillance. The World Health Organization (WHO) has stated the need for cross-border surveillance systems that can promptly identify a large variation in emerging disease outbreaks.[13] In situations where complex surveillance is difficult for logistic, technical or financial reasons (e.g., in certain developing countries), syndromic surveillance is a potential strategy.[14] A number of syndromic surveillance initiatives in public and animal health have been designed and implemented within the last decade with varying success for a wide range of gastric, respiratory and vaccine-preventable diseases.[15]

The pros and cons of syndromic surveillance
As a strategy, syndromic surveillance has a number of potential advantages and drawbacks. One advantage is that information can be provided without laboratory confirmation.[16] By use of pre-existing trend data, this general approach provides timely recognition of variations in syndromes related to a disease or group of diseases before laboratory confirmation.[17] An increase in clinically identified respiratory, neurological, and gastrointestinal syndromes heralded an increase in laboratory-confirmed cases of known causes of these syndromes in the Netherlands by a number of weeks.[17] Retrospective evaluation of known disease variation in accordance with syndromic data showed that up to 85% of variation in syndromes could be explained by known epidemics, especially if specific high-risk groups and local communities were monitored.[17] In **chapter 1** we demonstrated that this kind of syndromic surveillance can also be applied to travellers to provide information on disease activity in countries with limited surveillance data, as well as the risk to public health through travel. We showed evidence for disease activity via our traveller surveillance up to a year before information reached the WHO through the local health network. However, because this was a retrospective study, further investigation into the feasibility of prospective real-time surveillance is needed.
Monitoring syndromes may be more valuable than monitoring confirmed diseases. Syndromic surveillance systems are considered to be more sensitive to outbreaks of emerging diseases, especially when applied in real time.[10, 18] Emerging diseases present as clusters of cases that do not correlate to any known endemic or seasonal diseases.[17] In **chapter 2** we showed that monitoring of traveller-based syndromes can also be used for syndromic surveillance. We showed that the increase in patients returning from India with DENV-like symptoms without a laboratory diagnosis of DENV heralded the CHIKV outbreak in

the region. In **chapter 6** we found that Zika virus (ZIKV) had already become more active in travellers than previously thought. Although, arguably, our study population was too small to extend this claim to the traveller population at large, it provides evidence of previous ZIKV activity in travellers. Increasing the size of our traveller population could have provided a more substantial warning of extensive ZIKV virus activity. Given that humans form the main reservoir for ZIKV compared with DENV, CHIKV and YFV, earlier recognition of its outbreak potential and current increase in activity could have been achieved. Although there is no vaccine for ZIKV, its earlier recognition as an international public health threat could have stimulated earlier development and implementation of diagnostics and monitoring.
The potential downsides of syndromic surveillance are, first, that the increased sensitivity can come with a decreased specificity, since syndromic surveillance has been shown to result in more false warnings of potential outbreaks due to a lack of specificity.[14] Second, due to low numbers of cases in small communities, its sensitivity can diminish substantially when applied on only a local scale.[18] Therefore, a need still exists for a balance between loss of sensitivity due to monitoring in a small population and loss of specificity due to grouping too much data on a national level. Much effort has gone into tackling the problem of low specificity by developing improved algorithms to identify aberrations.[4, 19]
Additionally, challenges are associated with the use of syndromic surveillance of travellers for surveillance of the incidence of arboviruses in populations in general. First, the risks of acquiring local infections may differ for travellers compared to locals, as travellers may reside in luxury resorts and hotels. Second, our study focused on data retrieved from national diagnostic centres and flawed by extensive information loss during processing, thus reducing potential sensitivity (Figure 1). In **chapter 2**, we found with this data that little attention had been placed on defining specific goals and standardising definitions of syndromes and systems, frustrating the integration of these systems and thus decreasing the sensitivity.[17, 20] We have provided a number of methods to reduce this data loss, but if reduction is not achievable, real-time combining and monitoring of data from key travel clinics and tropical medicine hospitals would provide more timely, specific and sensitive data than are currently available.
Finally, challenges due in part to the fact that syndromic surveillance is a new field should be addressed by further research. These include: “defining optimal data sources, standardizing signal-detection methods, developing minimally acceptable response protocols and defining the use of simulation data sets to test systems”.[21] Currently, none of these challanges have definite solutions.
The unanswered question is whether syndromic surveillance in travellers is effective. If it is, what results can it be expected to yield?[21] If not, syndromic surveillance has nevertheless shown potential to be a flexible local, national and international surveillance method in terms of unknown or emerging diseases.[10,16] It provides the opportunity to adhere with limited economical resources to the WHO request for cross-border surveillance for an expanding list of diseases.

Filling in the gaps: developing a multiplex serological protein microarray

In **chapter 5.1** we focused on improving serological diagnostic methods because many arboviruses cause a short viremia, and thus 95% of results available for surveillance is serological (**chapters 2 and 4**). Therefore, the most logical step is a focus on increasing the quality and quantity serological diagnostic tests available. We developed an innovative multiplex protein microarray platform for serological diagnosis of flavivirus infections in

humans, while reducing serological cross-reactivity between closely related viruses and vaccines (**chapter 5.1**). Because only a small amount of viral antigen and serum was required, an extensive representation of multiple pathogens was possible per test. We extended the use of the microarray for serology in equines (**chapter 5.2**), since they are essential within an early warning system for current circulating and emerging flavivirus in Europe.

We showed that use of flavivirus NS1-proteins, produced in mammalian cell-lines as the main viral antigen, provided a more specific but still sensitive diagnostic method compared to current front-line diagnostic platforms like enzyme-linked immunosorbent assay (ELISA) and Indirect fluorescent antibody (IFA). However, further optimization of the test protocol for use in multiple species is still needed, as antibody reactivity, blood viscosity, interacting proteins, and possible nonspecific reactions due to other infections might require another species-specific approach.[22, 23]

To choose or not to choose ……. between diagnostics and surveillance

The development of new diagnostic tests, specifically for flaviviruses, is problematic.[24] Besides the extensive cross-reactivity between flavivirus antibodies, the context in which a diagnostic test is designed has a large role in its potential for risk of bias when used outside that context. Unlike development and marketing of new drugs, there are no compulsory guidelines for evaluation of diagnostic tests. In 2003, however, the Standards for Reporting of Diagnostics Accuracy (STARD) guidelines were published to fill this gap in quality assurance and have since been applied by many journals to all articles regarding diagnostics.[25]

The interpretation of test results differs depending on the purpose of the test (screening, surveillance or diagnostics), population in which it is applied (young, old, travellers, exposure history), or the disease prevalence in the population (endemic, sporadic or absent).[26, 27] Many diagnostic tests are developed in optimal in vitro settings where clearly positive samples containing large amount of antibodies serve as positive controls and known negative populations serve for determining specificity. As we showed in **chapter 5.1**, application of the microarray in travellers results in less interference from exposure history compared to application in residents of endemic countries. These results demonstrate the need to re-evaluate arbovirus tests according to the field setting when they are used for multiple goals.[26] If background information on usage and evaluation is not considered, inappropriate use or changes in diagnostic test methods can follow and can strongly influence surveillance data.[28] This variation in results can be seen in **chapter 5.2**, where we applied the microarray in equine populations for a) an experimental setting, b) diagnostics and c) surveillance in areas endemic for flavivirus, and d) surveillance in areas non-endemic for flavivirus.

Diagnostic tests have comparable but different objectives of sensitivity and specificity from surveillance tests, and the objectives can be inversely correlated.[26, 27] From a patient-care perspective, a missed diagnosis due to lack in sensitivity could result in invasive, unnecessary and expensive testing. From a surveillance perspective, specificity is essential for identification of the ecology, risk factors and related control schemes. Shifting cut-offs to lower specificity, however, could arguably improve identification of closely related emerging virus species or strains of geographically, clinically and genetically constantly changing arboviruses. Therefore, although the best solution would be to achieve both optimal sensitivity and specificity for both diagnostics and surveillance, a possible solution would be

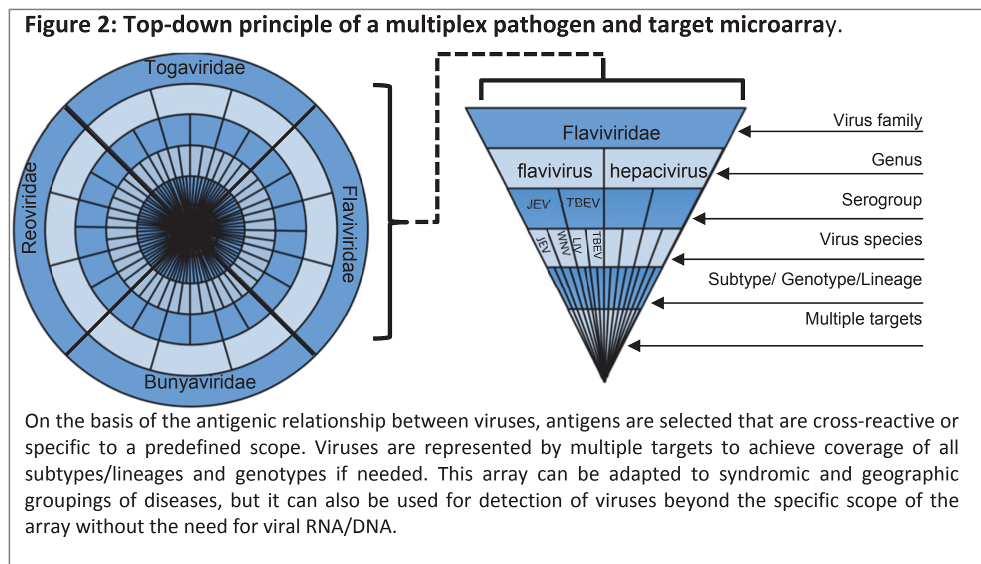
to test pathogens on multiple specificity levels using less to more conserved antigens and epitopes (Figure 2). In our current microarray we achieved better specificity for both patient care and surveillance without substantial loss of sensitivity by multiplexing pathogens and using a more specific antigen. A future challenge would be to multiplex the pathogens as well as the number of targets per pathogen and to increase the sensitivity without loss of specificity (Figure 2). The microarray platform offers this possibility because of its extensive multiplexing capability and easy adaptability to syndromic, geographic and antigenic questions.[29]

Putting the microarray to the test: a retrospective study of travellers

To further evaluate the potential added value of the systematic approach to travel diagnostics, we devised a study (**chapter 6)** to retrospectively test groups of patients at risk of flavivirus infections (according to the criteria in **chapter 3.1**) by quantitative reverse transcription polymerase chain reaction (RT-PCR) and with the multiplex serological protein microarray.

Through application of standardized flavivirus diagnostics by RT-PCR and microarray according to travel history, the number of patients identified with a recent flavivirus infection increased from 12% (52/436) to 19% (82/436). Eighty-five percent (70/82) of flavivirus infections were indicative of a DENV infection; 16 out of 84 patients tested by RT-PCR were found to be positive for DENV, and 13 were previously undiagnosed with DENV. Additionally, evidence of a wider range of flavivirus infections in travellers was detected (Japanese encephalitis virus (JEV), WNV, ZIKV, YFV). An internationally standardized diagnostic algorithm and use of multiplex techniques have shown potential improvement in both travellers' health and surveillance, although further optimisation is needed for better results.

Figure 2: Top-down principle of a multiplex pathogen and target microarray.



On the basis of the antigenic relationship between viruses, antigens are selected that are cross-reactive or specific to a predefined scope. Viruses are represented by multiple targets to achieve coverage of all subtypes/lineages and genotypes if needed. This array can be adapted to syndromic and geographic groupings of diseases, but it can also be used for detection of viruses beyond the specific scope of the array without the need for viral RNA/DNA.

But who's paying?

This broader diagnostic perspective is proposed to add financial and personal value for the individual patient by identifying potential complications, reducing expensive and invasive additional testing, and potentially using experimental therapies for virus-specific relief. However, how many negative tests must be performed, and at what cost, to diagnose one additional case? To answer this question requires an economic calculation that takes into account all the financial saved costs and benefits to global health.

With vaccine-preventable diseases, calculations often focus on the healthcare costs of an outbreak rather than the costs of surveillance and vaccination. Studies into cost-effectiveness of hepatitis B virus (HBV) and hepatitis C virus (HCV) surveillance have shown that such calculations heavily depend on upcoming medical therapies and available pre-travel vaccines.[30] Similarly, in the field of arboviruses, DENV vaccines are currently becoming available or are in the registration phase.[31]

However, vaccines are unavailable for many other arboviral diseases, and for most, only supportive therapy is possible after infection. Specific calculations regarding saved costs due to correct diagnosis are lacking, possibly due to the assumption that no benefit can result from diagnosis due to the absence of a cure and the self-limited nature of many infections. Although lack of a cure is currently true, there is no evidence that diagnosis in infected patients does not have any added patient-care or financial value. Early identification and prevention of specific, severe complications allow for specific beneficial supportive therapy and restrict unneeded, expensive and invasive testing to determine a diagnosis. However, from a cost-benefit perspective, the question still remains to what extent the diagnostic algorithm should be implemented.

With a commonly used cost-benefit analysis, however, it is difficult to calculate the financial advantage of public health surveillance in preventing disease outbreaks as it is difficult to calculate the actual costs of outbreaks, specifically for emerging diseases. Large disease outbreaks can result in huge economic loss amounting to billions of dollars due to closing of trade or transport, loss of working days and reduced income from the tourist industry, as was seen in the severe acute respiratory syndrome (SARS) outbreak in 2003.[32] Including these economic factors in the equation shows that enhanced surveillance systems could easily be cost-effective, assuming such system
s could prevent these outbreaks. Careful calculation of the impact of available intervention schemes in past outbreaks will need further in-depth analysis to accurately predict the potential value of investing in surveillance of emerging diseases.

Currently, this role for arbovirus surveillance is limited to individual clinicians actively and retrospectively forwarding information to health facilities. These facilities have an important social responsibility to identify clinical abnormalities that could impact public health. Combining LIMS data and structuring information to facilitate practical interpretation of data for surveillance somewhat relieves doctors of paperwork, while allowing them to maintain their responsibility. Structurally expanding the pallet of tests, on the basis of current information, offers social value through real-time surveillance data. The questions are how much these data are worth and how we can obtain the information practically and at acceptable cost. From a surveillance perspective, the negative test results correlated with syndromes can provide as much information on emerging disease outbreaks as positive test results (**chapter 2**). The cost-benefit analysis must include these patient-care and surveillance aspects.

Conclusion

The goal of this thesis was to analyse the use of existing medical information and health structures for arbovirus surveillance in the Netherlands. We demonstrated that syndromic studies of existing information can be used to monitor the effectiveness of current arbovirus surveillance methods. Although preliminary, our study also showed that currently available health structures and medical information could provide essential additional information that, if approached appropriately, could improve arbovirus surveillance. To achieve this, serological and syndromic data should be interpreted with a multipathogen perspective. However, our test results were limited to an increase in mainly DENV-positive patients, with a limited increase in other flavivirus diagnoses.

Nevertheless, application of standardized flavivirus diagnostics performed with RT-PCR and microarray according to travel history showed potential in the identification of patients with a higher risk of a recent flavivirus infection. Although further optimization of the algorithm is required (as can be seen by the low yield) and thorough cost-benefit analyses for syndromic multiplex surveillance are not yet available, we showed that existing medical information combined with standardized flavivirus diagnostics and multiplex techniques can be used for arbovirus surveillance in the Netherlands, potentially improve both travellers' health and surveillance.

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Summary

Arboviruses are a group of viruses that use arthropod vectors, like mosquitoes, ticks and midges, as their main transmission route. Most pathogenic human arboviruses are sustained in a transmission cycle in which the vectors are mostly mosquitoes, and the animal reservoirs are mainly domestic poultry, wild birds, and rodents that serve as the amplifying hosts. Recent decades have seen increasing arboviral outbreaks caused by travel and trade, animal migration and spread of exotic vectors.

Current arbovirus surveillance in Europe is patchy and often focussed on a single disease. Also, it is typically separate from the general health structures, as the information systems and data used in the medical field are not optimal for use in surveillance. Given the diversity and unpredictability of emerging diseases and the difficulty of identifying which will be a future threat, what is required are innovative preparedness strategies and instruments to focus on multiple pathogens, species, and symptoms.

The goal of this thesis was to study how existing medical information and health structures can be used for arbovirus surveillance in the Netherlands. In devising such a strategy and tools needed, this thesis integrated travel medicine with public health surveillance, encompassing a global health perspective on arbovirus surveillance. We used travellers as sentinels for arbovirus activity and developed a microarray that allows for fast, cheap and accurate testing of multiple arboviruses simultaneously.

The overall project consisted in five interrelated steps. First, we investigated the suitability and usability of information from routine diagnostic databases for use as an information resource for surveillance of arboviral disease burden in travellers. We found that compliance to fill out minimal data between each step along the chain was the main point at which data could be lost. We showed that nevertheless trends in arbovirus diagnostic requests and results can be identified and correspond partially to reports of outbreaks of the disease in question.

On the basis of an extensive review, we then quantified which arboviruses pose a travel risk and how they can be categorized to provide systematic information for diagnostics and surveillance. The review systematically combined and structured the current knowledge on medically important travel-related arboviruses. It provided the basis for a standardized diagnostic algorithm and illustrated the necessity of a detailed patient history (e.g. travel history, symptoms experienced, vaccination history, engaged activities, onset of symptoms), to guide the diagnosis.

Thirdly, we investigated how arbovirus diagnostics are currently performed in the Netherlands and to what extent travellers are at risk of under-diagnosis. We showed that the current range of viruses travellers are tested for is incomplete and likely many more people carry these kinds of diseases than are diagnosed. As these diseases pose potential public health threats, physicians should be more aware of the arboviruses that travellers could be infected with, and protocols are needed regarding what infectious diseases physicians should check for when travellers present with particular symptoms.

The fourth step consisted of developing an arbovirus protein micro-array for multiplex serological diagnosis of arbovirus infections in humans (import via travellers) and equines (local circulation). For this we used flavivirus, a genus that holds many of the world's most prevalent arboviral diseases that are also considered the most important travel related arboviral infections. In most cases, flavivirus diagnosis in travellers is primarily based on serology, since the amount of virus remaining in a patient's blood is often low and typically has already been reduced to undetectable levels when symptoms set in and patients seek medical attention. We showed that it is possible to deal with the most prevalent problems associated with serological diagnostics. Our multiplex protein micro-array using recombinant NS1 proteins detected flavivirus antibodies while less affected by cross-reactivity among the different flavivirus species.

Our last step was to evaluate the added value of multiplex testing of arboviruses for public health surveillance and patient care on the basis of standardized algorithms, for which we did a retrospective analysis. For this, we used our previously developed syndrome-based diagnostic algorithm to identify which previously tested patients were likely to have had a particular arboviral infection based on the syndromes they presented with and their travel history. We then retrospectively tested these patients, independent of previous diagnostics performed, using the multiplex protein micro-array that we had developed. This had a number of results. We showed that current, non-standardized diagnostic algorithms result in underdiagnosis of flavivirus infections in travellers. Also, our standardized diagnostics was much more accurate in predicting which patients might be infected. Thirdly, our microarray was successful in actually diagnosing those patients with a flavivirus infection (including those that had been missed by the non-standardized test).

A further test involved horses, since these often serve as sentinels for active virus circulation in serological surveillance programs. This test showed that the NS1-microarray can potentially be used for diagnosing and distinguishing flavivirus infections in horses, and for public health purposes within a surveillance setting. Combined with a standardized, syndrome-based diagnostic algorithm, the microarray thus allows for fast, cheaper, syndrome-based laboratory testing for multiple viruses simultaneously, for veterinary and public health purposes.

The critical factors that still need to be addressed are the further optimization, standardization and internationalization of diagnostic algorithms and further integrating routine diagnostic results of travellers into public health surveillance programs. Although further optimization of the algorithm and microarray is required (as can be seen by the low yield) and thorough cost-benefit analyses for syndromic multiplex surveillance are not yet available, we showed that existing medical information combined with standardized flavivirus diagnostics and multiplex techniques can be used for arbovirus surveillance in the Netherlands, potentially improving both travellers' health and surveillance.

Samenvatting

Arbovirussen zijn virussen die overgedragen worden door geleedpotige vectoren. De meeste pathogene arbovirussen voor de mens worden in stand gehouden in transmissiecycli waarin de vectoren meestal uit muggen bestaan en waarin de dierlijke reservoirs meestal bestaan uit pluimvee, wilde vogels en knaagdieren die dienen als versterkende gastheren. In de afgelopen decennia is er een groei in het aantal arbovirusuitbraken veroorzaakt door reisbewegingen, handel, diermigratie en door de verspreiding van exotische vectoren.

De huidige arbovirussurveillance in Europa is vaak fragmentarisch en richt zich vaak op één enkele ziekte. Daarnaast gebeurt surveillance los van ziekenhuizen en diagnostische laboratoria, omdat de informatiesystemen en gegevens die door ziekenhuizen en diagnostische laboratoria gebruikt worden niet ontworpen zijn om data te aggregeren en standaardiseren voor surveillance doelen. Vanwege de verscheidenheid en onvoorspelbaarheid van opkomende ziektes en de moeilijkheid om vast te stellen welke een toekomstige bedreiging vormen, zijn innovatieve voorbereidingsstrategieën en instrumenten nodig die op meerdere pathogenen, soorten en symptomen tegelijk focussen.

De doelstelling van dit proefschrift was te onderzoeken hoe al aanwezige medisch informatie binnen onze gezondheidszorg gebruikt kan worden voor arbovirussurveillance in Nederland. Toewerkend naar een dergelijke strategie en dergelijke instrumenten werden in dit proefschrift reizigersgeneeskunde en public health surveillance geïntegreerd; een global health benadering binnen arbovirussurveillance. We aggregeerden en analyseerden data uit ziekenhuisinformatiesystemen en gebruikten reizigers als sentinels om arbovirusactiviteit te monitoren. Daarna ontwikkelden we een microarray die snel, goedkoop en nauwkeurig testen op meerdere arbovirussen tegelijk mogelijk maakt.

Het project bestond uit vijf onderling samenhangende stappen. Allereerst onderzochten we of informatie uit bestaande diagnostische databases geschikt en bruikbaar was als informatiebron voor surveillance van de arboviruszielktelast onder reizigers. We stelden vast dat het nakomen, door artsen, van de verplichting om minimale data in te vullen bij de verschillende onderdelen van verzoeken om diagnostiek het belangrijkste punt was waarop gegevens verloren konden gaan. We lieten zien dat desondanks op basis van de diagnostische verzoeken en resultaten trends konden worden vastgesteld die gedeeltelijk corresponderen met gerapporteerde uitbraken van de desbetreffende ziekte.

Op basis van een uitgebreide review kwantificeerden we vervolgens welke arbovirussen een gevaar voor reizigers vormden en op welke wijze deze gecategoriseerd konden worden tot een systematische informatiebron voor diagnostiek en surveillance. De review combineerde en structureerde op systematische wijze de bestaande kennis over medisch belangrijke reisgerelateerde arbovirusen. Het bood de basis voor een gestandaardiseerd diagnostisch algoritme en illustreerde de noodzaak van gedetailleerde anamnese van de patiënt om de diagnose te kunnen stellen, zoals reis- en vaccinatiegeschiedenis, ondernomen activiteiten, syndromen (ziektebeelden) die zich voorgedaan hebben en op welk moment.

De derde stap bestond uit een onderzoek naar de wijze waarop arbovirusdiagnostiek momenteel in Nederland uitgevoerd wordt en naar de mate waarin reizigers kans lopen op

onderdiagnose. We toonden aan dat het huidige spectrum van ziektes waarop patiënten getest worden te gelimiteerd is en dat waarschijnlijk veel meer mensen deze soort van ziekten bij zich dragen dan er gediagnosticeerd worden. Omdat deze ziekten een potentieel gevaar vormen voor public health, zouden artsen beter gewaar moeten zijn van de ziekten waarmee reizigers geïnfecteerd kunnen zijn, en zijn er protocollen nodig die voorschrijven op welke ziekten artsen dienen te testen wanneer patiënten bepaalde syndromen hebben.

Ten vierde ontwikkelden we een microarray voor multiplex serologische diagnose van arbovirusinfecties in mensen (import via reizigers) en paarden (lokale circulatie). Hiertoe gebruikten we flavivirussen, een genus met veel van ’s werelds meest voorkomende arbovirusziektes die tevens beschouwd worden als de belangrijkste reisgerelateerde arbovirale infecties.
In de meeste gevallen gebeurt flavivirusdiagnostiek op basis van serologie, omdat de hoeveelheid in het bloed van de patiënt overgebleven virus doorgaans reeds te laag is om nog gedetecteerd te kunnen worden op het moment dat symptomen zich voordoen en patiënten zich melden bij een arts. Wij toonden aan dat het mogelijk is de meest gebruikelijke problemen met serologische diagnostiek te vermijden. Onze multiplex microarray, die gebruik maakt van recombinante NS1-eiwitten, detecteerde flavivirussen en onderscheidde tussen verschillende flavivirussen en door vaccinaties veroorzaakte vals-positieve resultaten. Bovendien was het minder gevoelig voor kruisreacties tussen de verschillende flavivirussoorten.

De laatste stap bestond uit het beoordelen van de toegevoegde waarde van het multiplex testen voor public health surveillance en patiëntenzorg op basis van gestandaardiseerde algoritmes. Hiertoe deden wij een retrospectieve analyse. Voor die analyse gebruikten we het syndroom-gebaseerde diagnostische algoritme dat wij ontwikkeld hadden om vast te stellen welke eerder geteste patiënten een grote kans hadden op een arbovirale infectie, op basis van de syndromen die zich bij hen voorgedaan hadden en hun reisgeschiedenis.
We testten deze patiënten vervolgens retrospectief, onafhankelijk van de eerder verrichte diagnostiek, met gebruik van de door ons ontwikkelde multiplex eiwit-microarray. Hieruit volgden een aantal bevindingen. We toonden aan dat de huidige, niet-gestandaardiseerde diagnostische algoritmen leiden tot onderdiagnose van flavivirusinfecties in reizigers. Daarnaast was onze gestandaardiseerde diagnostiek veel accurater in het voorspellen van welke patiënten waarschijnlijk geïnfecteerd waren. Ten derde was onze microarray in staat die patiënten te diagnosticeren die een flavivirusinfectie hadden gehad (inclusief die patiënten die gemist waren door de niet-gestandaardiseerde test).

Een vervolgtest betrof paarden, aangezien deze in serologische surveillance programma’s vaak dienen als sentinels voor actieve viruscirculatie. Deze test toonde aan dat de NS1-microarray mogelijk gebruikt kan worden voor de diagnose en vaststelling van flavivirusinfecties in paarden en voor public health doeleinden in een surveillance setting. Gecombineerd met een gestandaardiseerd, syndroom-gebaseerd diagnostisch algoritme stelt de microarray dus in staat tot snelle, goedkopere en syndroom-gebaseerde laboratoriumtests voor meerdere virussen tegelijk, voor veterinaire en public health doeleinden.

Kritische factoren die nog geadresseerd dienen te worden zijn de verdere optimalisatie, standaardisering en internationalisering van diagnostische algoritmes en het verder integreren van gebruikelijke diagnostische resultaten van reizigers binnen public health surveillance programma's. Daarnaast dienen degelijke kosten-batenanalyses beschikbaar te komen voor syndroom-gebaseerde, multiplex surveillance. Ondanks dat verdere optimalisatie van onze algoritme en microarray noodzakelijk is toonden we aan dat de huidige beschikbare medische informatie gecombineerd met een standaard algoritme en multiplex diagnostiek gebruikt kan worden voor surveillance van arbovirussen in Nederland.

Curriculum Vitae

N.B. (Natalie) Cleton, Dierenarts

Natalie is geboren op 12 mei 1983 en tot haar achtste getogen in Durban, Zuid-Afrika. Na het behalen van haar tweetalig VWO-diploma werd ze via decentrale selectie tot de studie diergeneeskunde toegelaten en verhuisde naar Kanaleneiland (Utrecht). Tijdens de studie was ze al erg betrokken bij volksgezondheidsvraagstukken en heeft zij samen met medestudenten een interdisciplinaire studievereniging opgezet gefocust op volksgezondheid, waar studenten en professionals met een achtergrond in Geneeskunde, Diergeneeskunde, Biologie, Farmacie en Communicatie elkaar vonden. Haar onderzoeksstage heeft ze uitgevoerd bij de Raad voor Dieraangelegenheden (onderdeel van het toenmalige Ministerie van Landbouw, Natuurbeheer en Voedselveiligheid) op het gebied van diergezondheid en welzijn in de biologische varkens- en pluimveehouderij onder begeleiding van Laurens Hoedenmaker en dr. Frank van Eerdenburg.

Naast haar studie Diergeneeskunde heeft Natalie in de nog niet ingevulde avonduren een Master Applied Ethics afgerond. Hier zocht ze vooral verdieping in ethische conflicten die ontstaan door de interactie tussen dierenarts en samenleving. Haar afstudeerscriptie schreef ze onder begeleiding van dr. Ludo Hellebrekers (toenmalig voorzitter van de Koninklijke Nederlandse Maatschappij voor Diergeneeskunde) en dr. Frank Meijboom over dit onderwerp.

In 2010 is Natalie afgestudeerd als dierenarts met specialisatie Landbouwhuisdieren en Volksgezondheid. Vanwege een aantal succesvolle beursaanvragen kon zij voor een half jaar naar de Verenigde Staten vertrekken om te werken als Veterinary Research Fellow aan Colorado State University, Department of Veterinary Medicine and Biomedical Sciences in Fort Collins, Colorado. Ze werkte hier aan verschillende onderzoeken naar hoog-pathogene zoönotische infectieziekten. Onder begeleiding van prof. dr. Richard Bowen focuste ze zich uiteindelijk op onderzoek naar de pathogenese van West Nile Virus en Japanse encefalitis in wilde en gedomesticeerde vogels.

Na terugkomst in Nederland in 2011 is Natalie gestart met haar PhD bij het RIVM binnen de afdeling Emerging Infectious Diseases en bij het Erasmus MC, afdeling Viroscience. Binnen haar PhD ontwikkelde ze gecombineerde laboratorium- en epidemiologische strategieën voor surveillance van vector-gebonden virussen in reizigers. Daarop volgend werkte ze mee aan een aantal internationale projecten over arbovirussen bij mens en dier in Europa. In 2016 rondde Natalie haar promotiewerkzaamheden af tegelijk met een specialisatie Veterinaire Microbiologie.

Begin 2016 is ze begonnen als Associate Project Leader vaccinontwikkeling bij MSD Animal Health in Boxmeer binnen de afdeling Ruminants Biologicals R&D.

PhD Portfolio

Summary of PhD training and teaching

Name PhD student: N.B. Cleton
Department: Viroscience

Period: 2011-2016
Promotor: Prof. Dr. M.P.G. Koopmans
Co-Promotor: Dr. C.B.E.M. Reusken

1.Training	Year
Courses	
- Scienion training on for the sciFLEXARRAYER	2015
- Partek Course on Microarray and NGS (Molmed, Erasmus MC)	2014
- Advanced immunology (UU, Graduate School of Life Sciences)	2013
- Prevention, Management and Diagnostics of Arboviruses (ISGlobal, Barcelona)	2012
- Virology (Molmed, Erasmus MC)	2012
- Public health by numbers: epidemiology and biostatistics (EdX, Harvard University, online course)	2012
- Meta-analysis (RIVM course)	2012
- Biosafety level 3 training (RIVM course)	2012
- Applied statistics I: An introduction to probability with a view towards applied statistics (RIVM course)	2012
- Analysis of serological data (RIVM course)	2012
- Introduction to "R" (RIVM course)	2011
- Applied "R" (RIVM course)	2011
- SPSS and basic statistics (Molmed, Erasmus MC)	2011
- Global Public Health (NIHES, Erasmus summer school)	2011
Workshops and seminars	
- PhD weekend (Proneri, RIVM)	2015
- Research integrity (Proneri, RIVM workshop)	2013
- Communication (Proneri, RIVM workshop)	2013
- Negotiation (Proneri, RIVM workshop)	2013
- Communicating with Power and Influence (Proneri, RIVM workshop)	2014
- AiO-meetings RIVM, IDS-seminar RIVM	2011-2015
Oral and Poster Presentations	
- Oral presentation: *Medisch belangrijke arboviruses in Europa* (Nederlandse vereniging voor Medische Microbiologie, Amersfoort, The Netherlands)	2015
- Oral presentation: *Syndromic approach to diagnostics of febrile travellers as a basis for surveillance of emerging arboviral diseases* (Arbovirus refereeravond, Rotterdam, The Netherlands)	2015
- Oral presentation: *Medisch belangrijke arbovirusinfecties in internationale reizigers* (Havensymposium, Rotterdam, The Netherlands)	2014
- Oral presentation: *Syndromic approach to diagnostics of febrile travellers as a basis for surveillance of emerging arboviral diseases* (International Meeting on Infectious Diseases, Vienna, Austria)	2014
- Poster presentation: *Cross-species multiplex microarray for serological detection of flavi-, phlebo- and alphaviruses* (International Congress of Infectious Disease, Cape Town, South Africa)	2014
- Oral presentation: *Arboviruses in Europe, an increasing threat* (European Congress of Virology, Lyon, France)	2013
- Oral presentation: *Validation of a flavivirus protein microarray for simultaneous detection of and differentiation between West Nile, Japanese encephalitis, Usutu and dengue virus immunoglobin G and M antibodies.* (European Congress of Virology, Lyon, France)	2013
- Poster presentation: *Routine diagnostic data of sentinel travelers as a source for monitoring and surveillance of emerging arboviral diseases.* European (Congress of Virology, Lyon, France)	2013
- Oral presentation: *Clinically important vector-borne diseases of Europe* (Congress of the International Society of Travel Medicine, Maastricht, The Netherlands)	2013
- Oral presentation: *Travelers as part of an arbovirus sentinel surveillance system; a feasibility study*	2013

using 10 years of routine dengue diagnostics data (Scientific Spring Meeting Koninklijke Vereniging voor Microbiologie - Nederlandse vereniging voor Medische Microbiologie, Papendal, The Netherlands)

- Poster presentation: *The use of diagnostic databases for arbovirus monitoring and surveillance; a feasibility study with a focus on dengue virus. (*15th Annual Meeting of the European Society for Clinical Virology and European Society for Veterinary Virology, Madrid, Spain) 2012
- Oral presentation: *Case Report: Tick-borne encephalitis in two Dutch travelers returning from Austria. (*14th Meeting of the Internal Working Group of Tick-borne Encephalitis, Vienna, Austria) 2012
- Oral presentation: *Age related susceptibility of young poultry to Japanese encephalitis virus.* (6th European Meeting of Viral Zoonoses, St. Raphael, France) 2011
- Poster presentation: *Assessing the anamnestic data provided with arbovirus diagnostics requests to evaluate the dynamics of disease risks in Dutch travellers.* (6th European Meeting of Viral Zoonoses, St. Raphael, France) 2011

(Inter)national conferences

- PREPAREing for(Re-)Emerging Arbovirus Infections in Europe (ESCMID Postgraduate Course, Greece 2016
- Scientific Autumn Meeting - Nederlandse vereniging voor Medisch Microbiologie, Amersfoort, The Netherlands 2015
- One Health Conference, Amsterdam, The Netherlands 2015
- Havensymposium – Tropen aan de Maas, Rotterdam, The Netherlands 2014
- International Meeting on Infectious Diseases, Vienna, Austria 2014
- International Congress of Infectious Disease, Cape Town, South Africa 2014
- European Congress of Virology, Lyon, France 2013
- Congress of the International Society of Travel Medicine, Maastricht, The Netherlands 2013
- Scientific Spring Meeting Koninklijke Vereniging voor Microbiologie - Nederlandse vereniging voor Medisch Microbiologie, Papendal, The Netherlands 2013
- 15th Annual Meeting of the European Society for Clinical Virology and European Society for Veterinary Virology, Madrid, Spain 2012
- 14th Meeting of the Internal Working Group of Tick-borne Encephalitis, Vienna, Austria 2012
- 6th European Meeting of Viral Zoonoses, St. Raphael, France 2011

2. Teaching

Lectures

- Serology course for Medical Microbiologist (VUMC, Amsterdam, The Netherlands) 2016
- Associate scientific organizer, presenter and workshop co-leader: PREPAREing for(Re-)Emerging Arbovirus Infections in Europe (ESCMID Postgraduate Course, Greece 2016
- Epidemiology of arboviruses in travellers (Erasmus MC, summer course) 2015
- Workshop on Vector-bone disease surveillance in the Netherlands (RIVM, Bilthoven) 2015
- IDS-VIR: surveillance van arbovirussen (RIVM, Bilthoven) 2015
- Rotterdams arbovirus overleg en refereeravond (Erasmus MC) 2012-2015
- IDS-VIR: arbovirussen in reizigers (RIVM, Bilthoven) 2014
- Workshop on research integrity (Proneri, RVIM) in collaboration with Utrecht University and RIVM Academy 2014
- Risk assessment and surveillance of arboviruses (Erasmus MC, summer course) 2013
- Arbovirus surveillance (Erasmus MC, summer course) 2012

Supervising

- 6 month training in serological techniques, multiple technicians, Erasmus MC, Rotterdam 2015
- 5 month internship pre-PhD student, Rotterdam 2015
- 9 month internship BSc Laboratory Science student Hoger Laboratorium Onderwijs Utrecht 2015
- 6 month internship MSc Infection & Immunity student University of Utrecht 2014

List of Publications

INTERNATIONAL JOURNALS

Humphrey J. M., Cleton N.B., Reusken C.B.E., Glesby M.J., Koopmans M.P.G., Abu-Raddad L.J. The Epidemiology of Dengue, Chikungunya, and Yellow Fever in the Middle East and North Africa: Systematic Review and Meta-Analysis. (*PLoS Negl Trop Dis.*)

Cleton N., Wagenaar J., Vaart E. van der, Eijk A. van de, Reimerink J., Reusken C., Koopmans M. Syndromic approach to diagnostics of febrile travelers as a basis for surveillance of arboviral diseases. *PLoS Negl Trop Dis. 2015 Sep 15;9(9):e0004073.*

Reusken C., Cleton N., Medonca Melo M., Visser C., GeurtsvanKessel C., Bloembergen P., Koopmans M., Schmidt-Chanasit J., van Genderen P. Ross River virus disease in two Dutch travellers returning from Australia, February to April 2015. Euro Surveill. 2015 Aug 6;20(31)

Cleton N., Reusken C., Godeke GJ., Reimerink J., Koopmans M., et al. Spot the Difference: A protein microarray for geographical and syndrome based surveillance of flavivirus infections. *PLOS Neglected Tropical Diseases, 2015 Mar 13;9(3)*

Cleton N., Bosco-Lauth A., and Bowen R.A. Age related susceptibility to Japanese encephalitis virus in Indian runner ducks and white leghorn chickens. *Am J Trop Med Hyg.* 2014 Feb;90(2):242-6

Cleton N., Reusken C., Murk JL., de Jong M., Reimerink J., van der Eijk A. and Koopmans M. Using routine diagnostic data as a method of surveillance of arboviral infection in travellers: A comparative analysis with a focus on dengue. *Travel Med Infect Dis.* 2014 Mar-Apr;12(2):159-66.

Cleton N., Koopmans M., Reimerink J., Godeke GJ. and Reusken C. Come fly with me: review of clinically important arboviruses for global travelers. *J Clin Virol*. 2012 Nov;55(3):191-203

Reusken C, Reimerink J, Verduin C, Sabbe L, Cleton N and Koopmans M. Case report: tick-borne encephalitis in two Dutch travellers returning from Austria, Netherlands, July and August 2011. *Euro Surveill*. 2011 Nov 3;16(44): 96

LOCAL JOURNALS

N.B. Cleton, C.B.E.M. Reusken, E.C.M. van Gorp. [The chikungunya epidemic in the Caribbean: implications for travellers and physicians]. *Ned Tijdschr Geneeskd*. 2014;158:A7918.. [Dutch]

N. Cleton, M Koopmans, M. Braks, C. van Maanen en C. Reusken. [Possible Japanese encephalitis in Southern-Europe]. *Tijdschr Diergeneeskd.* 2014 Jul 1;139(7):20-5. [Dutch]

C. Reusken, I. Zutt, M. Kik, N. Cleton, J. Rijks, J. Schmidt-Chanasit, A. Gröne, M. Koopmans, J. IJzer. [No evidence for Usutu virus as cause of mortality in passerine birds in the Netherlands in autumn 2012.] *Tijdschr Diergeneeskd.* 2014 Mar 1;139(3):28-30. [Dutch]

N. Cleton, M Koopmans, C. Reusken. [Medically important arboviral infections in international travellers.] *Tijdschr Infect.* 2013;(8)6:185-1. [Dutch]

N. Cleton, M. Koopmans, M. Braks, C. Reusken. [Possible Japanese encephalitis in Southern-Europe] *Ned Tijdschr Med Microbiol.* 2013;(1)21:7-11. [Dutch]

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