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Epidemiological Aspects and Microbiological Characterization of Fevers among Residents of Mozambique and Swedish Travellers Returning from the Tropics

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Front page: drawing made by my daughter Cecilia, 10 years old.

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Epidemiological Aspects and Microbiological Characterization of Fevers among Residents of Mozambique and Swedish Travellers Returning from the Tropics

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Birgitta Lesko

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Trots allt…

Tack vare er tre, min älskade familj.

Mikael och våra fina barn Johan och Cecilia.

ABSTRACT

The common theme and aim for this thesis was to explore the epidemiology and the diagnostic possibilities of patients with non-malarial febrile infections of tropical origin, with both the individual patient perspective and the more general public health aspects in focus. Analysis from two study areas, Sweden and Mozambique, are presented in the project.

The infectious disease panorama in Mozambique has to a large extent been a blind spot. Further epidemiological studies, aiming at more knowledge to guide decisions on preventive measures, are needed. We performed two prospective investigations. The first one was a pilot sero-epidemiological study on vector-borne viral zoonoses, in which we screened serum samples from patients attending a health care clinic in the suburb of the capital Maputo. In the analysis we found that 29% of the patients screened had an antibody response against one or more of the viral pathogens. Our conclusion, based on these results, was that exposure to chikungunya virus (CHIKV), dengue virus (DENV) and Rift Valley fever virus (RVFV) had taken place, and that these viruses are circulating in the country. The second study was an investigation of the DENV outbreak in the cities Pemba and Nampula. We analysed serum samples for DENV by PCR from patients seeking medical attention for fever during 2015-2016. The results including PCR positive samples, serotyping and sequencing of strains, confirmed that DENV serotype 2 is now endemic in northern Mozambique.

In the Swedish multicentre study we prospectively included febrile travellers returning to Sweden from tropical areas defined as malaria endemic. Epidemiological data from questionnaires and clinical diagnoses given to patients by their attending doctors were first compared with results from a panel of extended laboratory diagnostics, primarily on convalescent samples. We then focused on the possibilities for early diagnostics with PCR, particularly for cases where no relationship between the febrile illness and a microbial pathogen had been identified. We also developed a universal PCR for diagnosis of early phase DENV infection. This universal single probe real time RT-PCR for DENV was then used for the DENV analysis on acute samples. The results showed that infectious disease clinicians in Sweden, when taking care of febrile travellers returning from the tropics, were in general able to establish a diagnosis based on laboratory diagnostics for relevant pathogens. That being said, we also noted that 30 % of the patients included in the study were dismissed with the diagnosis fever of unknown origin. It was also apparent from the results that influenza virus infection was a frequent, and often missed, diagnose among febrile travellers, regardless of the time of year. The antibody screening also identified several additional cases of dengue infection. When including the PCR for DENV in the diagnostic kit, it was possible to reduce even further the number of cases with diagnosis of unknown fever. This confirms that the universal PCR for DENV is a sensitive, specific and valuable diagnostic tool to use during the first 5 days in the acute phase of illness. Apart from influenza and DENV, Rickettsia and Leptospira infections stood out as differential diagnoses that needed to be addressed.

LIST OF SCIENTIFIC PAPERS

I. Helena H. Askling*, Birgitta Lesko*, Sirkka Vene, Angerd Berndtson, Per Björkman, Jonas Bläckberg, Ulf Bronner, Per Follin, Urban Hellgren, Maria Palmerus, Karl Ekdahl, Anders Tegnell, Johan Struwe
Serologic Analysis of Returned Travelers with Fever, Sweden
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II. Erik Alm, Birgitta Lesko, Gunnel Lindegren, Clas Ahlm, Sandra Söderholm, Kerstin I. Falk, Nina Lagerqvist
Universal Single-Probe RT-PCR Assay for Diagnosis of Dengue Virus Infections
PLOS Neglected Tropical Diseases, 2014, Vol 8, p e3416

III. Eduardo Samo Gudo*, Birgitta Lesko*, Sirkka Vene, Nina Lagerqvist, Sandra Isabel Candido, Nilsa Razão de Deus, Félix Dinis Pinto, Gabriela Pinto, Vanessa Monteiro, Virginia Lara Evaristo, Nilesh Bhatt, Ivan Manhica, Kerstin I. Falk
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IV. John Oludele, Birgitta Lesko, Isabel Mahumane Gundane, Fernanda de Bruycker Nogueira, Argentina Muianga, Sadia Ali, Flora Mula, Imelda Chelene, Kerstin I. Falk, Flávia Barreto dos Santos, Eduardo Samo Gudo
Dengue virus serotype 2 established in northern Mozambique (2015-2016)
American Journal of Tropical Medicine and Hygiene. *In press*

V. Birgitta Lesko, Nina Lagerqvist, Anders Tegnell, Sirkka Vene, Kerstin I. Falk
Unknown fever among travellers to the tropics- possibilities for early detection with PCR
Manuscript.

**authors contributed equally*

CONTENTS

LIST OF ABBREVIATIONS

Arbovirus	Arthropod-borne virus
BSL	Biosafety level
CDC	Centers for Disease Prevention and Control
CHIKV	Chikungunya virus
cDNA	Complementary DNA
DENV	Dengue virus
DENV 1-4	Dengue virus serotyp 1-4
DNA	Deoxyribonucleic acid
ECDC	European Centre for Disease Prevention and Control
ELISA	Enzyme-linked immuno sorbent assay
IFA	Immunofluorescence assay
IgG	Immunoglobulin G
IgM	Immunoglobulin M
JEV	Japanese encephalitis virus
MAT	Microscopic agglutination test
MicroIF	Indirect mikro immunofluorescence
NS1	Non-structural protein 1
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase PCR
RVFV	Rift Valley fever virus
SMI	Swedish Institute for Infectious Disease Control, now integrated in the Public Health Agency of Sweden
VFR	Visiting friends or relatives
WNV	West Nile virus
ZIKV	Zika virus

DEFINITIONS

Antibody titer	Measurement of how much antibody has been produced, the dilution of the sample (in a serial dilution) still giving a positive result
Antigen	A foreign substance or a microbe that when entering the body stimulates an immune response
Communicable disease	A disease that can be transmitted from for instance person to person, or from animals to humans
Endemic	Natural to or characteristic of a specific place or people
Epidemiology	Study of diseases in populations
Homologous	Derived from or developed in response to organisms of the same species
Incidence	The occurrence of new cases of a particular disease in a population within a specified period of time
Outbreak	Increased number of cases of an infectious disease in a certain geographical area during a period of time. A sudden rise in the incidence of a disease
Phylogenetic clade	Evolutionary relationships. A clade is a group of organisms with a common ancestor and all its lineal descendants
Prevalence	The percentage of a population that is affected with a particular disease at a given time
Vector	An insect or other organism that transmits a pathogen
Zoonotic disease	A disease that can be transmitted between animals and humans

1 AIMS AND OBJECTIVES

The aims of the thesis were to explore the epidemiology and the diagnostic possibilities of patients with non-malarial fever of tropical origin, with both the individual patient perspective and the more general public health aspects in focus. The specific objectives were as follows:

1. Analyse and describe the spectrum of infectious diseases brought to Sweden from the tropics with febrile returned travellers.

2. To perform an extended analysis of blood samples from clinical cases in Sweden with unknown or unspecified infectious aetiology after travel.

3. Analyse the seroepidemiology against zoonotic viral infections in residents of Mozambique.

4. Contribute to the methodological development of laboratory procedures for early detection of pathogens in endemic countries as well as among returning travellers.

2 INTRODUCTION

2.1 BACKGROUND

International travelling of today is intense, providing a channel for communications and positive interactions for people all over the world. On the other hand, travelling also provides a channel for human infections to spread rapidly all over the globe (1-3). Furthermore, apart from international travel, also other factors such as climate changes, international trade and exchange of goods (4), are ways by which infections could expand their geographical area of endemicity. Particularly when vectors such as mosquitos play a role in the spread of the infection, vectors being organisms transmitting a pathogen. The current challenge for communicable disease prevention and control is to find a way to minimize these risks and to protect the population against the spread of communicable diseases. And to do it without undermining the sustainability and prospects of increased world-wide interactions. In this respect, it is essential to consider the effects of control measures, not only on travellers, but also on those living in endemic countries.

The infectious disease panorama in tropical countries is vast. In tropical areas, and when defined as malaria endemic parts of the world, malaria remains important. But the fact is that a wide number of patients seeking medical attention for fever in the tropics are negative when tested for malaria (5). In spite of this, further diagnostic investigations are not always done, leading to frequent cases of unclear diagnosis (6). Investigating these alternative diagnoses, particularly looking at patients with fever of unknown origin, is one of the objectives of this thesis.

2.2 MICROBES AND DIAGNOSTICS

The diagnostic challenges of fevers with tropical origin are many. They include the broad spectrum and similarity of clinical presentations of diseases such as malaria, dengue, chikungunya, leptospirosis and rickettsial infections. Also gastrointestinal infections, respiratory tract infections and septicaemia, can be difficult to distinguish from the more tropical associated diseases.

This thesis will focus on non-malarial causes of fever with tropical origin. Malaria is still a primary suspicion in fevers of tropical origin, causing a significant burden of disease. But its numbers have been diminishing over the last years. For example in regard to childhood mortality, a systematic review found that mortality for malaria under 5 years of age was reduced by more than 30% between 2000 and 2015 (7). According to the 2016 WHO report the number of malaria endemic countries has gone from 108 in year 2000 to 91 countries, with a reduction in incidence rate by 41% globally during the same period (8). Despite this remarkable progress, in large areas of the tropics, malaria still is a prime diagnosis to suspect and rule out in febrile patient.

In many tropical countries HIV infection and tuberculosis are also infectious diseases with a large public health burden. These diseases also need to be mentioned in this context, but will

not be explored further in this thesis. It should be noted that primary HIV infection is one of the differential diagnosis in the febrile patient, travellers and residents alike (9, 10). Additionally HIV infection in the advanced stage with an immunocompromised patient can predispose for other infections, such as tuberculosis (11).

When malaria has been excluded in the febrile patient, the differential diagnosis of non-malarial febrile diseases will have to be explored. Some authors even state that patients with a positive malaria finding should be further investigated for co-infections such as those caused by arbovirus (6).

Which pathogens to consider might vary, depending on information about clinical symptoms, and destination of travel or the tropical country of residence (12). In view of the symptomatology rarely being disease-specific, a more extended analysis might be needed. Apart from which disease to suspect and test for, another challenge is to have access to appropriate tools, allowing for correct diagnoses both in patients residing in endemic countries and in travellers returning home with fever after visiting such regions. Timing and type of samples available for diagnostics have to be considered when choosing the diagnostic methods, both in relation to the time of exposure and for how long a period the patient has been symptomatic (13). Furthermore, early diagnosis might be important in order to pinpoint diseases needing medical treatment and to prevent the severe clinical complications associated with a number of infections.

Thus the questions are:
Which pathogen(s) could be responsible for the febrile illness?
When investigating the cause of fever, can the pathogen still be found at the time the samples are collected or do we have to rely on indirect evidence from the patient´s immune response to the infection?

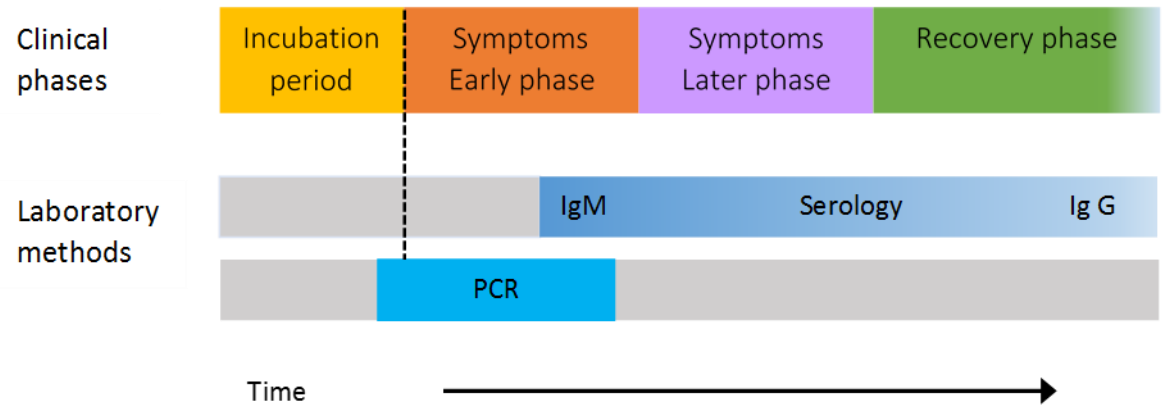

Figure 1. Clinical phases and schematic illustration of diagnostic possibilities with PCR and antibodies against the pathogen (IgM and IgG) over time. The figure is schematic, but could for instance illustrate the relationship over time between different diagnostic methods for arbovirus infection.

With arbovirus as an example, figure 1 shows that in the early phase of infection, even shortly before the patients have any symptoms, viral particles become present in the bloodstream. In the early stage, the pathogen can be diagnosed with PCR. A few days later, when IgM is being produced, testing for IgM antibodies against the pathogen becomes a diagnostic possibility. When more than 5 days have passed since the patient presented with symptoms, it is less likely to get a positive PCR result. Diagnostics after the acute phase will thus have to rely on the antibody response. In the later symptomatic phase and in the early convalescent phase the IgG antibodies against the pathogen will increase. The diagnostic method at this stage will then be comparing the IgG titer from the early symptomatic phase with the IgG level in the convalescent phase.

2.2.1 Detection of the pathogen

Direct detection of the microbe, or parts of the microbe, can be made from culturing the organism, microscopy, from molecular methods such as PCR, and by rapid tests for antigen detection. With direct detection you are actually able to see or identify the pathogen itself. These types of diagnostics are usually feasible in the early phase of an acute infection, just before symptoms have started or a few days after. The symptoms of the patient being an indirect indicator of the multiplication of the pathogen and the body´s response against the intruder. However in chronic infections the symptoms can be low-grade and the pathogen can be found for a prolonged period of time.

Culture

Culture can be performed on both viral and bacterial samples. Culture conditions and the possibility for a positive culture vary with the specific organism and the material collected. Some viruses and bacteria are more difficult to culture, and certain organism are classified as hazardous and have to be handled within a high-containment laboratory, i.e. a BSL-3 or BSL-4 facility.

Microscopy

Microscopy can be performed if you have a high enough concentration of organism. Electron microscopy (high resolution) is needed for visualizing virus-particles. Several species of bacteria can be seen in light microscopy after staining. Both clinical information and the use of additional methods might be needed for an accurate diagnosis.

Molecular methods

PCR (polymerase chain reaction) and NGS (next-generation sequencing) are molecular methods, analysing DNA or RNA, identifying organisms based on its nucleotide order.

Real time PCR

The PCR amplifies DNA. When preparing RNA it will therefore have to be transcribed by reverse transcriptase into complementary DNA (cDNA). The method measures the amplification of DNA after each cycle, using fluorescent dyes. In the one step real time PCR the reverse transcription is included, and the cDNA product is then amplified. The real time PCR amplifies distinct parts of the genome strand, defined by the sequences of the primers. It

is important that these are as specific and as unique as possible for the organism you are trying to find. The real time PCR is a qualitative method. If your aim is to measure the product, it needs a standard to be included to become quantitative.
The method is specific, positive results giving a diagnosis of high accuracy. With the new robotic extraction systems and thermal cyclers the analysis is fast, avoiding delays in diagnosis. The method has a low limit of detection, meaning that even a low amount of the pathogen can be detected. However the target regions for the primers and probes need to be sufficiently preserved. Genetic variations and new strains can develop. The specificities of the primers for the target RNA/ DNA need to be up-to-date and frequently checked against the genome sequences for new and different strains of the pathogen of interest included in a large genetic sequence database, e.g. GenBank, and necessary adjustments of the primer sequences made. The detection limits of the PCR should be known.

Next Generation Sequencing (NGS)
The method can sequence for instance whole virus genome. The first step of the NGS is a random fragmentation of RNA and/or DNA in the sample. Then the so called sequencing library is prepared, followed by amplification and sequencing. The data collected is then analysed in a computer and the results are interpreted (14, 15).

2.2.2 Detection of antibodies

Serology studies the immune response of the patient against the microbe, analysing blood serum for antibodies against the specific antigen. Detection of antibodies against the pathogen is an indirect method, not directly visualizing the pathogen itself. Furthermore, the antibodies can be the result of vaccination against the disease, and a single titer can then not differentiate between infection and vaccination. Despite this the methods can be specific, particularly when comparing titers in paired samples, the first sample taken during the early phase of the infection and the second sample during the convalescent phase.

Analysing immune response is a well-established method in the field of infectious diseases, using serological diagnostics of IgM and IgG titers. For interpretation of the results clinical information is needed, as the resulting titers are depending on when the samples were taken in relation to time of onset of disease. The antibodies are directed against the pathogen. The specificity of the response will depend on the type of antibody (IgM or IgG), the rise in titer (IgG), and the pathogen (cross-reactivity). In the acute phase of the infection, after a first time exposure to the pathogen, the IgM antibody is the earliest antibody to be found. It can be detected shortly after the symptoms have started, the titer rising over a couple of weeks, and then it vanes off. If IgM antibodies are found directed against a specific pathogen it clearly indicates the cause of infection. IgG titers take longer time before they are detectable in a first time infection with the specific pathogen. Once the IgG titers have risen, they usually stay on a high level for a longer period of time. If the patient is once more exposed to the pathogen, the level of IgG antibodies can rise rapidly. An acute, first time, infection can be verified by seroconversion or a four-fold or more increase in IgG titer, comparing antibody titers in two serum sample- one acute sample early on when the patient is presenting with symptoms, and

a second so called convalescent sample, preferably taken at least one month after the first sample. Studying the antibody response means that diagnosis can be made also when the acute stage of pathogen replication has passed, studying the immune response to the exposure. Cross-reactivity can be a problem, and is for instance common between flaviviruses (16, 17). It is thus important to now the vaccination status against for instance yellow fever and tick borne encephalitis. Non-specific reactions can occur in sero-diagnosis, and are important to consider when interpreting a positive result.

Some of the serological methods used for antibody detection are briefly presented below.

ELISA (Enzyme-linked immuno sorbent assay)
Several varieties of the ELISA assay exist. In the studies presented in this thesis we have used indirect ELISA assays in order to assess if there is an immune response (antibodies) in the patient sample against the pathogen. In this method, the wells in a micro titer plate are coated with antigen from the pathogen. The patient sample is added to the microtiter plate. After washing, a second labelled antibody against the presumed complex is added, as well as an enzyme substrate allowing for colour to develop if antibodies from the patient sample have bound to the pathogen. The colour reaction is then evaluated and read in a photometer plate reader.

Immunofluorescence assay (IFA)
Several different IFA methods are available, but here we present the method used for the studies and at the Public Health Agency of Sweden. Cultured cells are initially infected with the pathogen. The cells are then grown on 12-well slides to a confluent layer. Patient serum is serial diluted and added. After washing steps, fluorescein marked goat anti human IgG is subsequently added. The reaction is evaluated and the extent of cells with cytoplasmic fluorescence is estimated using a fluorescence microscope (18).

Agglutination
The agglutination methods are based on the fact that, when patient serum and bacterial antigen are added in a suspension, a clumping reaction will occur if antibodies against that specific, homologous, pathogen are present in the patient serum. Agglutination is used in the MAT test for leptospirosis (see below).

Neutralization tests are used in viral diagnostics. A neutralization test can be performed in several different ways, studying either the immune response or the presence of viral pathogens in a sample. An example of the method is the so called Plaque test (Plaque reduction neutralization test) where the presence of antiserum neutralizes the virus from infecting the cultured cells used in the test.

2.3 EMERGING ARBOVIRUSES

Vector borne diseases belonging to the group arthropod-borne viruses (arboviruses), are a group of viruses that have the potential to increase their geographical distribution and affect previously virgin human populations (19, 20). The arboviruses that are pathogenic to humans are all single-stranded RNA viruses. The clinically most important arboviruses belong to one of the four families Bunyaviridae, Flaviviridae, Reoviridae and Togaviridae, see examples in table below. The incubation period is usually short (3-10 days). The clinical picture range from a mild and self-limited febrile illness, maybe with a rash, to severe joint and muscle pain, haemorrhagic disease and encephalitis. It is often difficult to distinguish the different arboviral diseases merely based on symptomatology (21-23).

Table 1. Clinically relevant arbovirus of tropical origin

	CHIKV	DENV	RVFV	WNV	ZIKV
Family	Togaviridae	Flaviviridae	Bunyaviridae	Flaviviridae	Flaviviridae
Genus	Alphavirus	Flavivirus	Phlebovirus	Flavivirus	Flavivirus
Most common vector	Ae. aegypti Ae. albopictus	Ae. aegypti Ae. albopictus	Aedes spp. Culex spp. and other	Culex spp. Ae. atropalpus	Aedes spp. Ae. aegypti (Ae. albopictus)
Geographical distribution	Tropics-subtropics	Tropics-subtropics	Sub-Saharan Africa	Worldwide	Tropics - subtropics
Clinical symptoms	Fever, severe arthralgia, myalgia, rash	Fever, headache, arthralgia, myalgia, vomiting, rash. Haemorrhagic in severe cases	Fever, liver abnormalities, encephalitis, haemorrhagic fever	Fever, headache, body aches, nausea, vomiting. Encephalitis in severe cases	Fever, rash Neurological complications Congenital malformations

Different factors such as environmental change, travel or transport of goods, can facilitate the vectors expanding to new regions, providing the conditions necessary for these viruses to spread and increasing their risk of becoming a threat for public health. Arboviruses are often present in overlapping endemic regions coinciding with malaria, causing outbreaks of varying size and infections with similar symptomatology, challenging the diagnostic skills and possibilities (23, 24).

2.3.1 Chikungunya virus

Chikungunya virus (CHIKV) is an alphavirus. Sudden onset of fever and severe joint pain are clinical symptoms characteristic for CHIKV infection (25). It is a vector borne disease, transmitted by day-biting *Aedes* mosquitoes (26), particularly in tropical areas of Africa, Asia and the Indian subcontinent. It was first isolated in eastern Africa (Tanzania) in 1953 during an outbreak (20). Episodic outbreaks have occurred in Africa, India, and Southeast Asia,

spreading to Indonesia by the 1980s. In recent years it has spread to many subtropical countries, having been identified in 60 countries (25). During the last 10 years it has rapidly spread from what started as an outbreak in Kenya to the islands of the Indian Ocean and then also to the Americas. Following the wake of the large outbreak of chikungunya fever on islands in the Indian Ocean in 2006 (26-28), the fever was introduced in Italy by a returning traveller (29). There, it spread to local mosquitoes, the *Aedes albopictus* mosquito being already established in Southern Europe (30, 31). Later, in August 2007 a chikungunya outbreak was formally declared in Italy, for the first time in Europe (32). Being a virus that also can be transmitted by blood transfusion from an infected donor, these outbreaks pose a serious challenge to the health care system and to sustain blood supply (33).
Chikungunya virus now circulates in the same endemic areas as dengue virus and malaria, and the symptomatology for these diseases can be undistinguishable (20).

2.3.2 Dengue virus

Dengue virus (DENV) is a flavivirus with four related, but antigenically distinct serotypes. Infection with one of the serotypes 1-4 only confers immunity only to that specific serotype. Infection can present with a varying degree of symptoms (34), and differences in clinical symptomatology have in some settings been noted between serotypes (35, 36). Many persons with DENV infections have a mild, unspecific febrile illness, or may even be asymptomatic, and the disease resolves without complications. However in some cases severe disease develop, with bleeding, organ failure and shock, thus requiring urgent correct diagnosis and treatment (37). Severe disease outcome has been related to a second infections of DENV with another, heterologous, serotype (38, 39).

For most of the DENVs that cause human infection, humans are the only vertebrate host (both reservoir and amplification host) (20, 40). DENV is the most widespread arbovirus affecting humans and all four serotypes are now found in Asia, Africa, and the Americas (41-43). In the 1970s only nine countries were considered endemic for DENV. Now largely distributed in the tropical and subtropical regions of the world, currently there are more than 100 countries in Southeast Asia, Latin America, and the Western Pacific region affected by DENV. According to recent WHO estimates approximately 96 million persons present with symptoms of DENV infection out of the probably 390 million people that become infected with DENV per year. Ten times as many, 3.9 billion people, that are living in 128 different countries are according to WHO at risk of DENV infection (44). Several of the endemic countries are tourist destinations and visited by many travellers.

The vectors are the daytime biting mosquito *Aedes aegyptii* together with *Aedes albopictus* (30). The mosquitoes being present pose a risk for autochthonous transmission of DENV, that could when imported cause local transmission in southern Europe (45, 46), the vector *Aedes albopictus* being well-established on several locations (31). Local transmission of DENV has occurred in Europe. In 2012-2013 there was an outbreak of DENV-1 in the island of Madeira, with more than 1000 confirmed cases. *Aedes aegypti* , one of the vectors for DENV, is known to be present in Madeira since 2005 (47).

2.3.3 Rift Valley Fever virus

Rift Valley Fever virus (RVFV) is a phlebovirus belonging to the family Bunyaviridae. It is a viral zoonosis, first recognized 1912 in the Rift Valley in Kenya (48). Since then there have been several epizootic outbreaks mainly affecting livestock in Africa and the Arabian Peninsula, with the potential for worldwide spread. The epidemiology of RVF is complex, and from being a mild disease rarely affecting humans during the 20th century outbreaks, the number of human cases and the severity, including risk for mortality, have clearly increased (49). RVFV has been classified as a bioterrorism agent by the World Organization for Animal Health (OIE) (49, 50). RVFV is mainly transmitted by mosquitos of the *Aedes* and *Culex* species, but several different mosquitoes, flies and ticks can be vectors (49-52). Direct transmission from infective body fluids is also possible. In livestock it has been associated with congenital abnormalities and fetal death (49, 51). An increased risk of miscarriage has been reported in humans, studying RVFV infected mothers (53). In light of the ongoing ZIKV outbreak this raises concern for the effect it could have on pregnant mothers and their unborn child (49), especially if RVFV continuous to expand into new geographical areas.

2.3.4 West Nile virus

West Nile virus (WNV) is a flavivirus and one of the most widespread arboviruses, with several outbreaks involving humans and/or horses in Europe and in North America (16). It was not introduced into the USA until 1999, but then it spread rapidly (54-56). Birds play a key role in the cycle of the virus together with species of *Culex* mosquito being the main vector (57). Humans and other mammals are dead-end hosts (58). Infected humans are mostly asymptomatic (80%), but there is risk of neurological symptoms in less than 1% of the cases (West Nile neuroinvasive disease). WNV transmission has been known in Europe at least since the 1960s when it emerged in the Camargue in the south of France. Transmission occurs from time to time in Central European and Mediterranean countries (59). A public health implication is the risk of human to human transmission from blood transfusion of infected donors. During the 2010-2012 outbreak in North-eastern Italy, blood-donors in the affected provinces were screened through nucleic acid test (NAT) (16, 60). 43% of the reported WNV RNA-positive blood donors were asymptomatic (16).

2.3.5 Zika virus

Zika virus (ZIKV) is, similar to DENV and CHIKV, primarily transmitted by day-biting *Aedes* mosquitos. It is a flavivirus that was first isolated from a rhesus monkey in the Zika Forest of Uganda in 1947 (61). The virus was not found outside Africa and Asia until 2007. Then it was detected in an outbreak on a Micronesian island (61), the symptoms were described as mild and self-limited.

The ZIKV outbreak in Latin America is a recent example of the public health impact caused by an emerging arbovirus. When reaching a former unexposed large population in South America, it has had massive public health impact, particularly for women in childbearing age and especially in Brazil. Many questions on risk factors still remain to be answered (62).

The outbreak was a challenge for the health organisations, particularly in locations where the outbreak was new and ongoing. With the intense focus of attention on ZIKV infection the risk of other diseases being missed or misdiagnosed was apparent, causing an added health threat. In light of the ZIKV outbreak an increased number of cases of falciparum malaria in Venezuela and Colombia was reported (63). The same trend is believed to have occurred in other countries of the region as described in ProMED mail 27 Aug 2016 (64).

2.3.6 Mayaro virus

Mayaro virus (MAYV) is an alphavirus closely related to CHIKV (65), and was first isolated in Trinidad 1954. MAYV is endemic in South America (65), and causes a dengue like illness (20, 66). As for CHIKV the presence of arthritis might be a distinguishing symptom of alphavirus from other differential diagnoses (65). A case report in Europe has recently been published on a French citizen with a dengue-like syndrome and arthralgia after a travel in French Guiana (67). This was the 6th case of confirmed MAYV infection reported from Europe, but under-diagnosis among travellers is very likely, and the real incidence among travellers is not known. Even though mosquitoes of the *Culicidae* family are the primary vector there is concern that MAYV also could be transmitted by *Ae. albopictus* which already is established and expanding in Europe (67). This calls for increased awareness and attention on possible MAYV infections, leading to a correct diagnosis and timely preventive measures aiming at limiting spread of both the vector and the infection.

2.3.7 O'nyong-nyong virus

The o'nyong-nyong virus (ONNV) is an alphavirus, and the symptomatology is similar to CHIKV infection. ONVV seems to be restricted to Africa, with reported outbreaks in East Africa, but also reports from western Africa with cases from for instance Chad and Nigeria (65, 68, 69). There are single reports on cases in travellers returning to Europe, for instance from Chad or Kenya to Europe (68, 70). Humans might be the only vertebrate host. The virus is primarily spread by anopheline mosquitoes (71, 72).

2.3.8 Diagnostics of arbovirus

The symptomatology after arbovirus exposure is often unspecific, with several differential diagnosis to consider and causing a diagnostic challenge. The laboratory methods primarily used are antibodies against the pathogen and PCR.

PCR

In early acute stage of the disease, when we can assume that the febrile patient is viremic (73), molecular methods such as RT-PCR is a good choice for arbovirus detection (65). In acute dengue the duration of dengue viremia (73) has been shown to range from 2-12 days, but most patients had detectable circulating virus for 4-5 days. Sensitivity and specificity of the tests have to be considered, and for DENV diagnostics several specific and sensitive PCR-based assays have been developed including a DENV serotype-specific multiplex real-time RT-PCR approved by the US Food and Drug Administration (74). However most of

these assays need a large number of primers and/or probes to be able to detect all four DENV serotypes, complicating modifications when new genetic variants of DENV appear. Additionally, simultaneous circulation of new and old strains has been reported (75) and also spillover of sylvatic DENV to humans (76) requiring a wide range of strains to be covered for detection.
Also for the other arboviruses described above diagnostics with PCR technique is available.

Serotyping with PCR
PCR-based techniques can be used for determining the infecting DENV serotype during the early, 5-6 days, of DENV disease. Both multiplex and singleplex methods exist. Singleplex, serospecific real time PCR has the advantage that they can easily be modified if new variants of the DENV strains emerge (77). Sequencing can also be done to verify the specific serotype.

Nonstructural protein 1
Diagnostics of DENV can be made by detection of non-structural protein 1 (NS1), an early soluble viral antigen secreted by DENV infected cells (78, 79). As for viral RNA, NS1 can be found from the onset of disease (80). The NS1 Viral antigen peak is around day 3 post onset of fever. The NS1 protein may be detectable in serum for some days longer than the viral nucleic acid, which usually is present until at least day 5 (81-83). An NS1 ELISA was introduced in 2006, but the most used NS1 antigen test today is one of the commercially available rapid tests. However, the sensitivity of the rapid tests is low compared to the high sensitivity of the PCR (81). In secondary DENV infection NS1 is present during a shorter period of time, cross-reacting antibodies forming immune complexes (80). Furthermore, the NS1 tests have a lower sensitivity in secondary DENV infection compared to in primary DENV infection (81).

Serology
When it comes to serology of flavivirus there are some things to keep in mind. Cross-reactions in flavivirus serology are well-known and can occur either after previous flavivirus infections or after vaccination with for instance Yellow Fever vaccine. Thus it is important that the clinician reports the vaccination status of the patient when referring a sample for antibody analysis.

For DENV it is also essential to know if the patient has a primary or secondary infection with DENV, since the immune response will differ for the two. A primary infection of DENV is characterized by a slow and low-titer antibody responses. In general IgM and IgG antibodies will start to appear during the early post-febrile period (84), where the IgM response is dominating and can become present day 2 to 5 after onset of fever (80, 85, 86). IgG antibodies generally become detectable at the end of the first week of illness (41). In secondary infections IgG antibody titers rise rapidly while IgM antibody levels can remain low or undetectable (85).

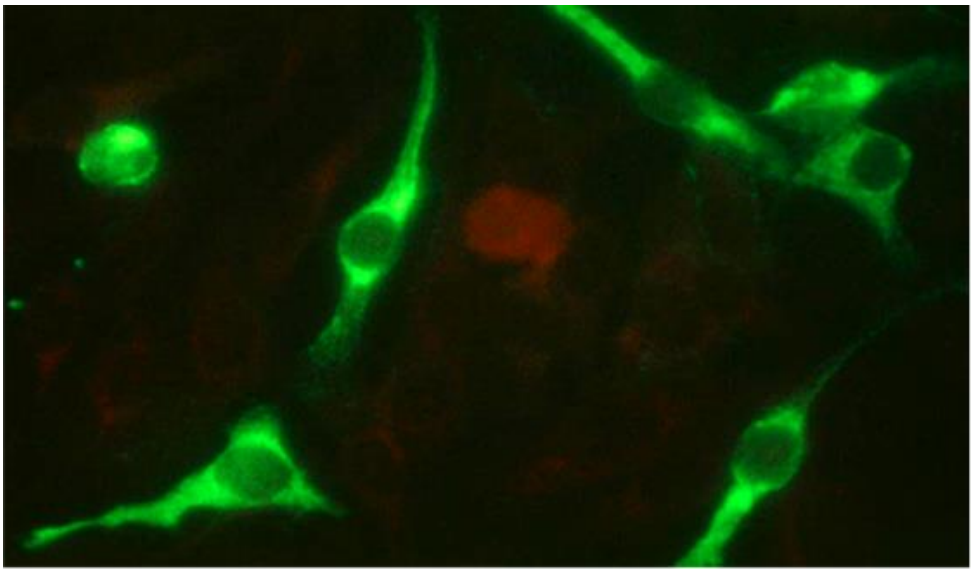

Figure 2. Positive indirect IFA for DENV. Kindly provided by Sirkka Vene

Laboratory confirmed diagnosis

A confirmed DENV case can be defined as having either a fourfold or more increase in paired samples of IgG antibody titer between the acute and the convalescent paired sample (24), a positive PCR finding for DENV, or the combination of a positive NS1 antigen and DENV IgM in the acute sera.

Similarly for other microbial pathogens a more than fourfold increase in IgG titers or a positive PCR result confirms the diagnosis, as does a positive culture or virus isolation.

Today, PCR is the best way to diagnose patients with suspected arbovirus infection (65), but maybe the future lies in new "screening" diagnostic methods for unknown febrile illnesses, such as the further development of the next generation sequencing (87), and broader but specific immunological methods for the later stage of viral diseases that is after the viremic phase.

2.4 OTHER TROPICAL VIRAL AND BACTERIAL PATHOGENS

A large number of viral and bacterial pathogens can of course cause infections of tropical origin, many of them also being common in other parts of the world. One such disease is HIV infection. Within this thesis only a limited number of pathogens have been further investigated, mainly to explore the possibility of them being neglected diseases in the diagnostic process.

2.4.1 Hantavirus

Hantaviruses are found worldwide and are important zoonotic pathogens. Two forms of hantavirus disease are seen in different parts of the world. In Asia and Europe haemorrhagic fever with renal syndrome or nephropathia epidemica can be seen, while in the Americas hantavirus infection is associated with a cardiopulmonary syndrome. Outdoor activities and inhalation of dust contaminated with rodent urine or other excretes, can be a risk of exposure (88). Over 50 species of hantaviruses have been identified worldwide, however up until 2006 none had been reported from Africa (89). Serology is still the golden standard for Hantavirus diagnostics. The primary differential diagnoses are leptospirosis (renal failure) and rickettsiosis (rash, and maybe pulmonary oedema, but the eschar distinguishes from hantavirus infection) (90).

2.4.2 Influenza virus

Influenza virus is a respiratory virus. The infection is a highly transmissible disease from one person to a non-immune individual. Due to the antigen shift and drift of the virus, immunity after infection is seldom protective. A virus strain that has changed enough in its antigen properties will have the chance to infect and spread. Influenza appears in cycles. The seasonality differs over the globe. In the northern hemisphere the yearly season for influenza typically peaks during the winter months (December-February) (91). Vaccines against influenza are available, and recommended for elderly and other risk groups. The vaccines are updated and adjusted according to the circulating strains of the virus, and vaccination needs to be repeated on a yearly basis. Influenza is mentioned as a neglected disease in febrile travellers and consider vaccination (92, 93).

2.4.3 Leptospira spp.

Leptospirosis is a zoonosis associated with exposure to soil and water contaminated by urine from animal carriers, mostly rodents, of the bacteria. Both domestic and wild animals can be affected. Research articles are mainly found on the veterinary field (94). Infected persons can present with mild symptoms mimicking other diseases, but the disease spectrum is wide and the mortality high in severe cases when icterus can develop. Outbreaks have been reported after flooding and storms, the majority of alerts coming from the Americas, followed by Western Pacific and South East Asia. Comparatively few alerts come from the African region (95), in spite of the fact that leptospirosis is endemic in the Sub-Saharan African region. Taking into account the available data on human infections, the disease can be considered a

neglected tropical disease in this region (94). Leptospirosis can occur among travellers, and have particularly been associated with adventure travels (96, 97).

Laboratory diagnostics of Leptospirosis are cumbersome, and often only available at reference laboratories. Several methods are used for the diagnosis of leptospirosis. Microscopic agglutination test (MAT) has a high specificity, detects both IgM and IgG antibodies and is said to be the gold standard serological test for early diagnosis of leptospirosis. The high specificity of the MAT test make it a useful tool for confirming the leptospirosis diagnosis, but the test requires culture of Leptospira strains with the risk of laboratory contamination and infections. In the early phase of the disease the sensitivity and specificity of the ELISA IgM is high, becoming positive 1-2 days earlier than the MAT. Detection of bacterial DNA by PCR is an alternative early diagnostic method and can also be used to confirm a case (94, 98). Blood for the PCR needs to be drawn preferably before or very shortly after antibiotic treatment is started. Otherwise the sensitivity of the PCR is drastically reduced, as the Leptospira will have been cleared from the blood by the antibiotics given (99).

2.4.4 Rickettsia spp.

Rickettsiae are strict intracellular bacteria. What is generally called rickettsiosis usually refers to two entities, *Orientia tsutsugamushi* (scrub typhus) and the genus *Rickettsia.* The *Rickettsia* can be further divided into the spotted fever group (SFG) rickettsiae (e.g. *Rickettsia conorii, Rickettsia rickettsii, Rickettsia africae)* and the typhus group rickettsiae (*Rickettsia prowazekii* and *Rickettsia typhi*) (100, 101). The vectors for the SFG ricketssiae are ticks, and several species that were previously thought to be non-pathogenic have now been reclassified and associated with human infection (101). Other arthropod vectors for rickettsial infections include lice and fleas. Clinical symptoms associated with rickettsiosis are fever, rash and generally eschar (102-104). Serology is still the most common used method for diagnosis (104), but antigens can cross react between different rickettsiae (101). The results of indirect microimmunfluorescens (MicroIF), using acetone fixed bacteria, needs confirmation and further analysis for a more detailed specification. PCR can be performed on eschar tissue or blood, although very few bacteria might be present in blood during the acute phase, as has been noted in reports on diagnostics of for instance Rocky Mountain Spotted Fever (105). This could be a limiting factor in PCR diagnostics of the acute disease. However, in severe cases it is interesting to note the PAN real time PCR for rickettsial disease was found to be more sensitive than previous used PCR methods (105). As in the case of Leptospira, another limiting factor when using PCR is if antibiotic treatment already has been started before taking the samples.

2.5 INTERNATIONAL TRAVELLERS AND DISEASE PATTERNS

Swedish travellers

People living in Sweden are in general relatively well protected from the spread of infectious diseases. Good hygienic conditions prevail and public health is well developed. Medical progress and preventative measures protect the population from infections that, in other regions of the world, may cause large scale disasters. Travel abroad among Swedish residents of today is intense and frequent (106). This means that infections can be brought back home when travelling to the tropics (107), and patients sometimes seek care presenting symptoms difficult to diagnose.

Current Swedish data on cases of imported tropical diseases is mainly provided by the SmiNet registry at the Public Health Agency of Sweden (108). This register contains reports on all cases of diseases, the so called notifiable diseases, included in the Swedish Communicable Diseases Act from 2004 (109). Statistical reports on aggregated data from the register is presented at the home page of the Public Health Agency of Sweden https://www.folkhalsomyndigheten.se/folkhalsorapportering-statistik/statistikdatabaser-och-visualisering/sjukdomsstatistik/.

One of the tropical diseases included among the notifiable diseases in Sweden is malaria, a vector born parasite. According to statistics, a decreasing number of malaria cases have been diagnosed in Sweden, with approximately 90 to 120 cases being reported annually. A sudden peak in the number of cases occurred during 2014, with 354 reported cases. The figures for 2015 went down to 249 cases and decreased further during 2016 to 154 reported cases. The years with comparatively high numbers in reported malaria cases, especially during 2014, corresponds in time to the influx of refugees and migrants from malaria-endemic countries. For dengue fever the number of cases have increased constantly over the last 10 year period, from around 50 to 60 cases registered 10 years ago to 225 cases during 2016. This is the highest number of DENV infection ever reported in Sweden. The variation might correlate to a change in travel patterns and other factors related to increased risks of infections in endemic countries. It might also correlate to the level of awareness among clinicians and the availability of diagnostic methods.

Other tropical diseases, like for instance chikungunya fever, are not even included in the list of notifiable diseases in Sweden. This means that the ability to identify trends in disease epidemiology followed by returning travellers or a large influx of migrant populations carrying the disease is limited, depending on whether or not patient samples are sent in for analyses to the laboratory.

Studies on travel related fever

When this research project started in 2005, few prospective studies on returning travellers investigating fever of unknown origin had been published (110-116). Studies with regard to travellers returning to the Nordic countries were particularly scarce (117). Other studies done had been either retrospective or limited to research reviews (118-121). Some other prospective studies were also initiated and published at the time (122-124).

Studies available before 2005 reported that 2-3% of the returning travellers presented at a health care clinic with a febrile condition (125, 126). Then studies were published indicating that more than 10% of the returning travellers had fever (93), and that fever often was the main reason for seeking medical advice (127). Furthermore, as many febrile illnesses have unspecific and overlapping signs and symptoms, the task of finding the correct and complete diagnosis was not easy (117). A substantial number, as many or more than 25% of the patients seeking medical advice after travel to the tropics, were left with an unspecified or unspecific diagnosis concerning microbial aetiology (110, 118, 122).

Identifying microbes and correlating them to risk factors for communicable diseases, could benefit preventive measures and travel advice aiming at reducing exposure when travelling to risk environments (128, 129).

2.6 MOZAMBIQUE AND INFECTIOUS DISEASES

Mozambique is a coastal country in the south eastern part of Africa. The population was estimated to be approximately 28 million people in 2015 (130), with an average life span of approximately 55.5 years and a literacy of 58.8% in the population 15 years or older (131).

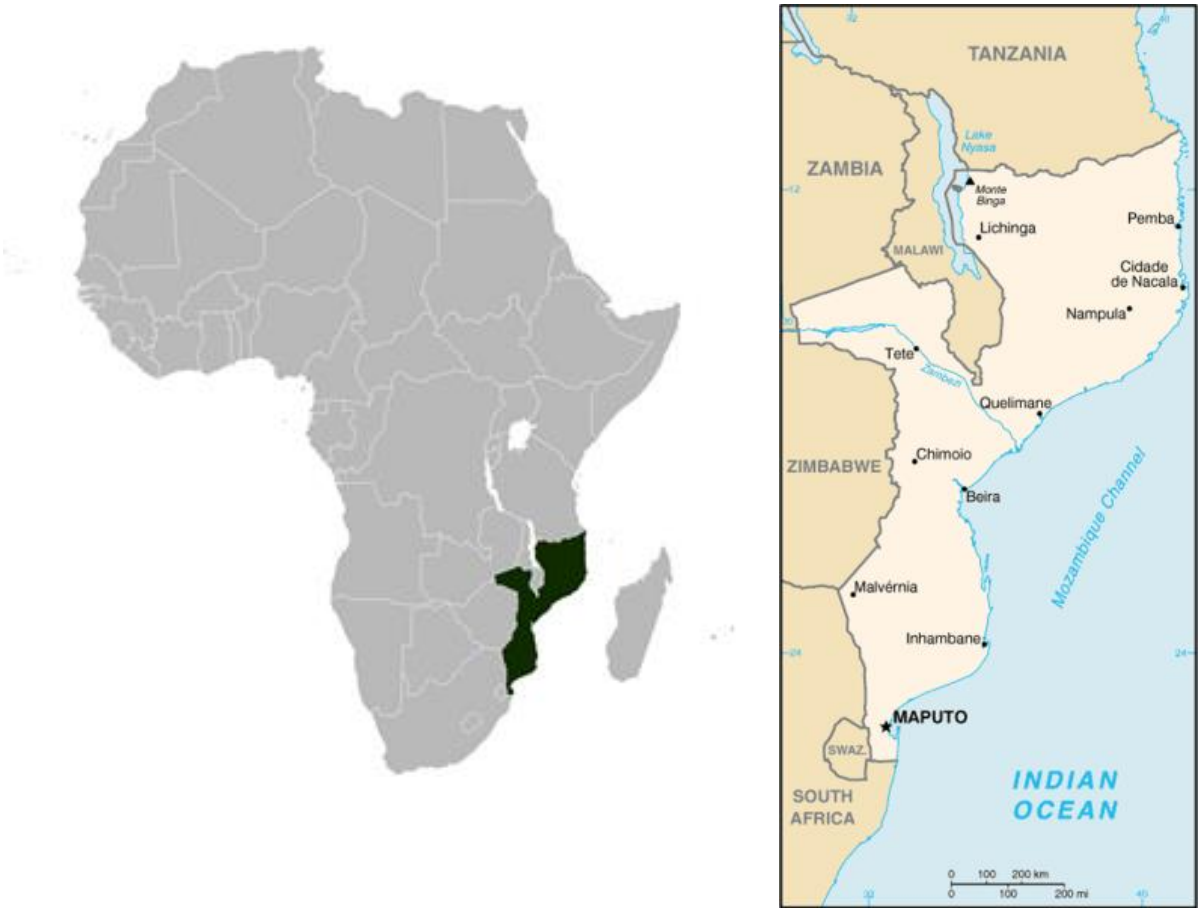

Figure 3. Maps of Africa and Mozambique.

Sources: By Shosholoza (Own work) [CC BY-SA 3.0 (http://creativecommons.org/licenses/by-sa/3.0)], via Wikimedia Commons and By CIA (CIA, The World Factbook, 2004) [Public domain], via Wikimedia Commons

Malaria and HIV/AIDS are the major causes of death in Mozambique (132). The HIV prevalence among adults was estimated in 2015 to be 10.5%. Approximately 1.5 million people are living with HIV, more than half of the infected persons now receiving antiretroviral treatment (133). Being a country with high prevalence of HIV, testing for HIV infection is often routinely performed on patients seeking acute medical attention. The number of notified cases of malaria and the number of deaths it causes are declining, but this disease still poses major public health problems. Malaria is still the most common cause of death, responsible for 35% of child mortality and 29% of the adult mortality, with more than 8.3 million notified confirmed cases and approximately 15000 deaths during 2015 (134, 135).

That being said, acute febrile illnesses are frequent in Mozambique and not all of them are found to be caused by malaria. Also other aetiologies need to be explored.

In recent years, CHIKV and DENV have caused several outbreaks and epidemics in the West Indies and the Americas, as well as in sub-Saharan Africa and Madagascar and in southern Europe (136-139). The importance of these pathogens has become more evident with time, as the burden of disease for malaria decreases in endemic areas (140). In fact a wide majority of the patients seeking medical attention for fever in the tropics are negative when tested for malaria (5). In spite of this, in Sub-Saharan Africa, including Mozambique, febrile patients are often assumed to have malaria and treated as such. Overlooking other diseases may lead to a risk of sub-optimal treatment and increased morbidity among febrile patients (6, 136, 141). The overdiagnosis of malaria and the inadequate diagnostics are therefore a cause of concern.

Further diagnostic investigations are not always done due to a lack of epidemiological data on alternative diagnosis to malaria in combination with poor laboratory capacity. Thus, viral zoonotic disease patterns among humans in low-income countries such as Mozambique have not yet been extensively surveyed. The number of reports published has been limited (142-144). One of the probably overlooked and underdiagnosed causes of infections in Africa is DENV (136).

A sero-epidemiological study conducted more than 30 years ago in Mozambique, found antibodies to RVFV in 2% of pregnant women (145). Studies on Rift Valley fever endemicity among cattle in the Maputo Province during 2010-2011 showed a sero-prevalence of 36.9 %, indicating that RVFV is prevalent among bovines (146). There has also been a movement of the human population from the rural areas to the major cities, particularly to the capital Maputo, with a large influx of residents to the suburban areas (147).The change in living conditions might have an impact on the spread of viruses as well as disease patterns since urbanization often leads to an aggregation of susceptible human hosts, frequently living under socioeconomic conditions promoting vector population expansion and at increased risk of arbovirus transmission (20).

Further studies and reports are now being published, giving background data on the spread of emerging viral diseases in the human population, providing a basis for future actions of prevention and intervention. In March 2015 the Health Ministry in Mozambique confirmed an ongoing outbreak of dengue fever in the northern province of Nampula while a previous outbreak in 2014 in a neighbouring province was contained (148). A recently published study from Mozambique on CHIKV infection found seroconversion or a four-fold increase in antibody response against CHIKV in 4.3% of the examined febrile patients, indicating that CHIKV is circulating in southern Mozambique (149).

2.7 PUBLIC HEALTH ASPECTS

Relevance for public health

Surveillance and knowledge about disease patterns constitute a foundation for public health actions aimed at preventing diseases at the population level (150). The spread of communicable diseases is often multifactorial. Apart from the risk of travel, other determinants of health can facilitate the spread of infections and thus affect public health. Such determinants of health are for instance international transport and trade between different parts of the world, global movement of populations as well as climate and environmental change (4). The effects of climate change on human health have recently been highlighted, stating that there is both a direct risk of outbreaks resulting from extreme weather events, but also an indirect risk even of slighter climate changes resulting in altered geographical distribution of vectors (151). Furthermore, the WHO and UN Health and Environment Linkages Initiative (HELI) encourages countries to address health and environment linkages, emphasizing prevention and control, while pointing at vector borne diseases as one of the priority risks (152).

The current general theory of epidemiological risk transition is linear and directed towards the relatively increased burden of disease caused by non-communicable diseases (153). When it comes to communicable diseases, one could argue that the epidemiological transition should not always be viewed as linear. It could be looked upon as having a certain trend due to public health actions, but still have the potential of regression and move back to an earlier stage if these actions are not sustained, spreading more widely and leading to outbreaks. There is indeed always a risk of reversed epidemiological transition, with re-emergence of infections considered to be under control (154-156).

Keeping communicable diseases in focus as a health risk enables timely introduction of public health interventions in the form of relevant preventive measures, particularly in endemic countries. But this focus is also applicable to travellers and in migrant populations. Evidence based public health is important, as it leads to interventions directed towards exposed populations with adequate and high quality information on prevention (157). Identifying microbes and correlating them to risk factors for communicable diseases remains therefore essential, not only for appropriate surveillance and correct diagnosis of infectious diseases but also for decision making on public health actions. The interaction between public health actions and individual prevention is obvious. Both perspectives need to be implemented for optimal outcome.

An example of the importance of keeping these to perspective in mind is the ZIKV outbreak in the Americas. This challenging situation had public health effects at a broader international scale, being recognized as a Public Health Emergency of International Concern in the WHO statement of the International Health Regulations Emergency Committee on ZIKV in February 2016 (158). In the initial stages of the outbreak, when many risks and risk factors were still unknown, public health authorities in many countries struggled with giving advice

and relevant recommendations due to the severe effects the disease could have on pregnant women and their unborn child. This was particularly true regarding worried travellers and pregnant mothers in despair, contemplating abortion (159). In Europe, the established exchange of information and synchronization of measures in accordance with EU legislation on cross border threats to health proved to be essential in this situation (160). The European Centre for Disease Prevention and Control (ECDC) played an important role in providing updated risk assessments as a tool for support, summing up facts, analysing risks for Europe and giving advice to Member States (161-163).

Consequences of preventive measures not being maintained

A significant reduction in the number of cases related to a disease not yet been completely eliminated, may lead to paradoxical and unexpected challenges for public health. When an infection is perceived to be successfully controlled and rarely occurs, the perception of the risks implied by the disease may also change. This could lead to a neglect or disregard of preventive measures. Individual and public health actions might not be thought of or be followed to a lower degree by the population. This lowered risk perception has for instance been noted when analysing insurance data and costs (164). Measles for instance is a disease that can easily be spread from country to country by non-immune travellers (2, 165), this fact should emphasize the importance of high vaccination coverage in any population against this disease. In spite of that, measles outbreaks still occur in Europe from time to time, mainly in pockets of low vaccination coverage but with the potential to spread to other areas where vaccination coverage is suboptimal (166-168). Other factors may also coincide and complicate the task of finding correct and complete preventive measures. Research findings show that some of the arboviral diseases, like CHIKV, seems to have a natural cycle with re-emergence and outbreak of the disease after a period of time (169). Thus the increased number of CHIKV cases in Asia during the 1960-70s was followed by a period of stabilization and decline before re-emerging in 2005.

Continued research will thus always be necessary to enable adequate and high quality preventive measures.

3 THE PRESENT STUDIES

Infectious diseases associated with residence in or travel to the tropics constitute a diagnostic challenge. A broad spectrum of febrile illnesses with unspecific and overlapping signs and symptoms makes advice and diagnosis to febrile travellers and residents of tropical areas particularly difficult for the clinicians involved.

The current thesis focuses on and explores the epidemiology and diagnostic possibilities in two contexts: Sweden and Mozambique.

Today´s international travelling is intense (170). Many travellers come back with fever, and in about 30% or more of the cases the microbiological diagnosis is unknown. Residents living in tropical countries, such as Mozambique, are also left with unclear diagnosis and are often presumed to have a malaria infection, without further investigations being carried out.

Since disease patterns and spread of infections are changing over time, it is of particular importance both to increase access to relevant epidemiological information and to provide reliable diagnostic methods that are easy to use for health care.

Study rationale

My research aimed at broadening the evidence base necessary for decisions on preventive measures and diagnostics, seeking to improve the tools for clinical investigation. Furthermore, the studies aimed at raising awareness of travel related tropical diseases.

The research cooperation with our colleagues in Mozambique opened for an increased and shared knowledge regarding the epidemiology of vector borne diseases. It also provided an added value in both our countries, Sweden and Mozambique. Learning from each other's experiences broadened our perspectives.

Papers presented

Two of the papers (I and V) are based on a Swedish multicentre study on febrile travellers returning from tropical areas of the world.

For two of the presented papers (III and IV) the study material is from Mozambique and the work has been done in cooperation with our colleagues there.

One paper (Paper II) describes the methodological development of a universal PCR for DENV.

4 MATERIAL AND METHODS

DATA SOURCES AND COLLECTIONS

Study areas and populations (Papers I, III, IV, V)

Papers I and V
Recruitment of adult patients with fever after travel to malaria endemic area, seeking medical attention at Swedish hospitals with an infectious disease department during 2005-2008. Informed consent was given at inclusion. The inclusion criteria were travel within two months prior to consultation, age 18 years or above, a documented temperature of 38°C or higher (oral, axillary or rectal) at admission or within the last two days (as reported by the patient) and the clinician's decision to perform a blood film examination for malaria. The catchment area covered 3.3 million inhabitants, constituting 1/3 of the whole population in Sweden.

Paper III
Febrile patients seeking medical attention at the Polana health centre, in the suburbs of Maputo, during 2012-2013 were included in the pilot study after informed consent. The catchment area was 4,663 km^2, with an estimated population of 46184 inhabitants.

Paper IV
Febrile patients seeking medical attention at the hospitals in the cities Nampula and Pemba 2015-2016 were included after informed consent. The recruitment area was Nampula and Cabo Delgado provinces in northern Mozambique, with a population of approximately 470,000 in Nampula and 140,000 inhabitants in Pemba.

Questionnaires (Papers I, III, IV, V)

Epidemiological information was collected in a questionnaire at the time of inclusion. For Paper I and V a follow-up questionnaire 1 month or later after the first visit was filled in by the clinician.

Blood samples (Papers I, II, III, IV, V)

In the studies for Paper I, III, IV and V, blood samples were collected after informed consent. The samples were anonymised and then analysed within the research project.

For Paper II serum samples from the biobank at the Public Health Agency were used in accordance with the assignment of the national authority – see 5.6 Ethical considerations.

LABORATORY METHODS

The laboratory methods used have been described in the background chapter of the introduction and are referred to in the results and discussion for the individual papers.

5 RESULTS AND DISCUSSION

5.1 PAPER I

Serologic Analysis of Returned Travelers with Fever, Sweden

The first paper is about Swedish travellers coming home from different tropical areas of the world. In this multi-centre study patients were enrolled at five of the Swedish hospitals with an infectious disease department. The paper describes epidemiological data and presents results from screening for antibody response against presumed pathogens in this group of patients.

During the period 2005-2008, 514 patients fulfilling the inclusion criteria were prospectively included after giving an informed consent. The material to be analysed included questionnaires and blood samples. For 383 patients we obtained a paired blood sample of acute serum and a follow up convalescent sample. For these patients with paired blood samples we choose to look for the most probable causes of fever and using the methods available at the time – that is approximately 10 years ago.

In the article we included two sets of patients. The multicentre study with prospective case finding included 514 patients, of whom 383/514 provided a paired sera. A retrospective case finding group of 918 patients was also included, based on a malaria test having been performed in the individual case. The analysis of the retrospective control group confirmed the representativeness of the material included in the prospective study.

The study analysis in the prospective group was based on the epidemiological analysis of the questionnaires, including the clinician's assessments of diagnoses, and results of antibody screening of the serum samples. Serology for influenza A and B were performed at the Department of Microbiology Malmö University Hospital. MAT and PCR for leptospirosis were undertaken at Statens Serum Institute (SSI) in Copenhagen, Denmark in order to confirm the positive findings at SMI for Leptospirosis. All other analyses were performed at the Swedish Institute for Infectious Disease Control (SMI). The pathogens in focus, for which all 383 convalescent samples were screened, were: Influenza A and B viruses, DENV, CHIKV, *Brucella spp*., *Leptospira spp*., *Coxiella burnetii*, *Rickettsia spp*. (Typhus group: *R.typhi, R.prowazekii*; and Spotted fever group: *R. africae, R.conorii*). When the travel destination was to Asia, serology for *Orientia tsutsugamushi* and Japanese encephalitis virus was added.

When analysing the questionnaires we found that the most common reported dismissal diagnoses reported by the clinicians for the 383 patients were "unknown fever" (30%), followed by gastroenteritis (24%), malaria (7.5%), pneumonia (5%) and septicaemia (5%). Influenza was diagnosed in 2% of the cases. The additional study serology found antibody response against a number of pathogens, adding an infectious disease diagnosis to cases that had been missed to diagnose in the clinical setting (see table 2). Particularly the number of influenza cases increased. After the additional serology, infection with influenza was the specified diagnosis most frequently found, with 9 % (34/383). Among the influenza cases, 25% had occurred outside the usual time frame for the northern hemisphere influenza season. A majority of these latter patients had returned from a trip to Africa.

Overall the study serology reduced the number of cases with fever of unknown origin from 115 to 91/383, a reduction of 21%. Positive findings also added a diagnosis of infectious disease illness (co-infection) to 23/283 patients already diagnosed with one infection. DENV co-infection was for instance found among cases already diagnosed with Salmonella gastroenteritis, Borrelia infection, or pharyngitis. Co-infection with influenza was found for pyelonephritis, salmonella sepsis, giardiasis, malaria falciparum parasitemia, pyelonephritis and erysipelas. A leptospirosis case was combined with pyelonephritis and rickettsiosis with malaria (P. malariae).

Table 2. Summary of results for the prospectively included febrile travellers

Analysis of 383 febrile travellers returning from the tropics	**Microbiological diagnosis given by the clinicians**	**Extended serology adding diagnosis**		**Total number diagnosed including additional serology**
		Adding a diagnosis to the cases with fever of unknown origin n=115 / 383 (30%)	Co-infection	n=383
Disease		patients diagnosed	patients diagnosed	patients (% of 383)
Influenza	8	12	14	34 (9 %)
DENV fever	11	3	3	17 (4 %)
Rickettsios	7	6	4	17 (4 %)
Leptospirios	1	2	1	4 (1 %)
Q fever	2	1		3 (0.7 %)
CHIKV fever			1	1
Fever of unknown aetiology still after additional serology		91 (24 % of 383)		

When summing up the data, we came to the conclusion that influenza was a frequent cause of fever among travellers returning from the tropics, regardless of the time of the year the travel had taken place. Other studies confirm that influenza is frequent among travellers (171) .We found that this infection was often missed in routine diagnostics of febrile travellers. The extended laboratory analysis also indicated rickettsia, dengue virus and leptospira as alternative infections to be considered.

Interestingly, 24% of the patients were still left with an unknown aetiology to their fevers. Further research to bring knowledge on the epidemiology and spectrum of diseases brought to Sweden by travellers returning from the tropics could be of relevance.

The advantage of the study is that it is prospective, allowing for the collection of blood samples and patient questionnaires. However, since the epidemiological part of the study is purely observational no analysis on for instance interventions can be done. Neither do we have a denominator to calculate prevalence in the overall group of travellers to the tropical destinations of interest. A confounding factor to the results received could be that some of the febrile illnesses may not be related to the travel abroad, but to exposure that could have taken place after the return home. It would have indeed been interesting to make a similar study on travellers already identifying them prior to their trip, and then upon arrival back home dividing them in control group and clinical cases depending on their clinical status. Performing such a study was unfortunately not feasible within this project, considering the time and resources available at the time.

5.2 PAPER II

Universal Single-Probe RT-PCR Assay for Diagnosis of Dengue Virus Infections

In the second paper we describe the development of a new universal one step real time-PCR for diagnostics of dengue virus infections regardless of serotype (1-4). The real time PCR assay has been designed on the basis of all complete genome sequences (n=3305) of the four DENV serotypes published so far.

DENV is often considered the most important arboviruses worldwide. It is probably causing about 50–100 million infections per year and poses a threat to 2.5 billion people in tropical and subtropical regions. The risk for travellers is obvious. To diagnose travellers returning from different parts of the world can be a diagnostic challenge. Reliable and easy to use methods for diagnosing DENV during the early acute phase of infection are of great value, no matter which part of the world the traveller has visited. The methods should be possible to use both in endemic parts of the world and in the home-countries of returning travellers. The test options for early detection of acute dengue infection are for instance NS 1 antigen, serology (IgM) and PCR.

To target all variants of DENV strains by PCR would be an advantage. This would then mean targeting the genome of all dengue virus serotypes 1- 4 simultaneously, including new and

old strains as well as spill over sylvatic DENV strains to humans, in a single probe PCR assay. To achieve this all the complete genome sequences of dengue virus serotypes 1, 2, 3, and 4 published in GenBank November 2013 (n= 3 305) were analysed. In this process we used the tool BLAST, Basic Local Alignment Search Tool, searching for regions of similarity between sequences. We found one large enough and highly conserved region (64 nucleotides), and this region is the target for the primers and probe. The universal PCR has been tested and evaluated in different ways. An example being the analysis of 60 saved acute blood samples from patients with known DENV infection (see Fig 4 page 7 in Paper II). The different patients had been having symptoms for a different number of days, ranging from 1 day to 9 days, when the blood samples were collected. The viral load in the samples in relation to how many days the patients had had symptoms is displayed. The results show a high sensitivity of the universal PCR method, detecting 100% of the cases during the first 5 days post onset of disease. On day 9, with only a low number of genome copy equivalent still present, more than 50% of the cases are found PCR positive. The high sensitivity for the universal DENV RT-PCR was again noted in 23/23 PCR positive samples among which only 20 were positive by the NS1 antigen test, while these three positive results were confirmed by the CDC DENV-1-4 RT-PCR Assay.

The universal RT-PCR method was found to have a high sensitivity and specificity for early detection of dengue virus infection, and allows to detect diverse strains of the virus. It is thus a quick and efficient way to diagnose all serotypes of dengue 1-4. To assure a continued accuracy the sequences need to be checked on a regular basis against the Gen Bank at NCBI (National Center for Biotechnology Information, U.S. government-funded national resource for molecular biology information). The method is based on a highly conserved region, and the continuous sequence update shows that it still takes everything in the database.

The main added value provided by the method is that it allows to detect diverse strains of the virus. In the article we suggest that this universal PCR should be used as a first choice diagnostic method as an alternative to NS1 during the first 5 days of symptoms consistent with dengue virus infection.

5.3 PAPER III

Sero-epidemiologic Screening for Zoonotic Viral Infections, Maputo, Mozambique

The third paper presents results from a pilot sero-epidemiologic screening for zoonotic viral infections (CHIKV, DENV, Hantavirus, RVFV and WNV) in Maputo, Mozambique. Only limited epidemiologic data on alternative diagnoses to malaria is available in Sub-Saharan low-income countries. In these setting, urbanization and social factors as well as international trade could affect particularly the epidemiology of vector borne diseases. For Mozambique no such data was available. We were eager to start investigating it, and choose to focus on zoonotic viral infections.

In this pilot study febrile patients were prospectively included (N=78) during 2012-2013. Paired acute and convalescent blood samples were collected and screened for IgG and IgM

response to zoonotic viruses. Results for the 23 patients with positive responses are shown in the table below. Real- time RT-PCR was performed on all 78 samples, investigating four pathogens (CHIKV, WNV, RVFV and DENV). All of the PCRs were negative.

Among these Maputo residents 23 of the 78 patients (29.5%) had a positive serology for one or more screened viral pathogens, indicating previous exposure to these viruses. Ig G against CHIKV is clearly the most prominent finding in this pilot study, with 19% positive samples. Two of the 10 DENV positive patients had a positive IgM test against DENV, indicating a current acute infection with DENV.

The positive IgG against WNV might be the response of cross reactivity within the flavivirus complex, since in two patients it was found together with positive IgGs for DENV and CHIKV, with the lower IgG titer against WNV. Cross reaction with antibodies against yellow fever vaccination is also a possibility.

One patient was positive in the IgG screening for RVFV. Further investigations and wider testing regarding RVFV might be warranted, when considering that an investigation in the late 1980s found antibodies against RVFV in 2% of pregnant women in Mozambique (145). Additionally a study in the Maputo province in 2010-2011 showed a sero-prevalence of 37% among cattle (146). These findings in combination with the results of this pilot study could be an indication of a potentially emerging RVFV infection among humans in Mozambique.

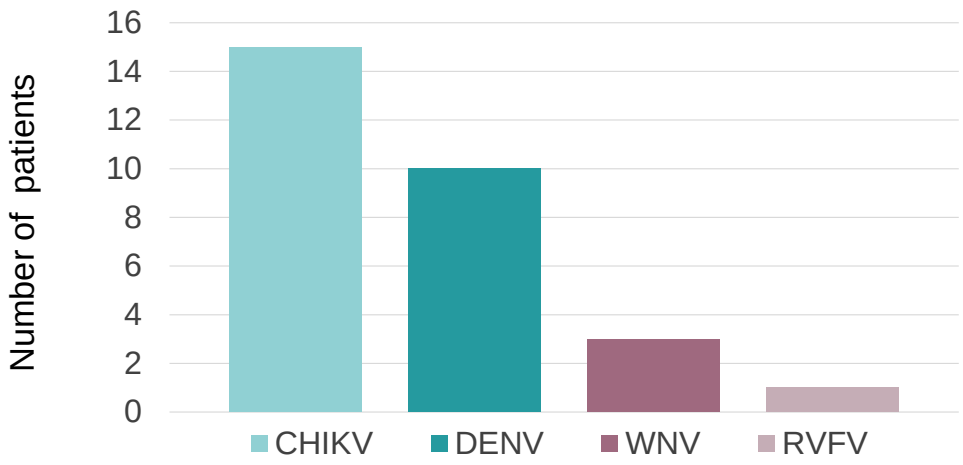

Figure 4. Positive findings in the screening for IgG and IgM response to zoonotic viruses

Table 3. Summary of positive results for screening against viral antibodies and microscopy for malaria parasites

Organism	Positive screening results Maputo residents n= 78	
	%	n
CHIKV	19.2 %	15
DENV	12.8 %	10
RVFV	1.2 %	1
WNV	3.8 %	3
Malaria parasites	19.2 %	15
Total positive serology against viral pathogen	29.5 %	23

A screening for malaria parasites was performed on all 78 included patients and 15 (19.2%) malaria positive patients were found. Three of the 15 malaria-positive patients had a positive serologic finding (2 for DENV IgG and 1 for CHIKV IgG).

This investigation shows that 29.5% of the included patients had a positive serological finding for one or more of the viral pathogens studied, an indication of previous exposure. The IgG results show that chikungunya virus, dengue virus and Rift Valley fever virus are circulating in Mozambique. These pathogens have therefore to be considered as differential diagnosis in febrile patients in the Maputo region. The main finding was IgG antibodies against chikungunya virus detected among 19% of the tested individuals. Another publication confirms the presence of CHIKV in Mozambique showing seroconversion or a four-fold titer increase in 4,3% of febrile patients (149). The positive Ig G result for CHIKV have recently been confirmed by NT test (personal communication with Eduardo Samo Gudo).

For RVFV a high prevalence among cattle in the Maputo region has been shown in 2010-2011, and it would be of interest to further explore the risk of exposure for humans.

5.4 PAPER IV

Dengue virus serotype 2 established in northern Mozambique (2015-2016)

Paper IV is a study on the ongoing outbreak of DENV infection in northern Mozambique. A previous DENV outbreak had taken place in Pemba 1984 to1985, and was also the first documented DENV-3 in Africa (142) The current outbreak started in 2014, again in the northern parts of Mozambique, involving the cities Pemba and Nampula (172). The aim of the study was to confirm the DENV serotype and to follow the trend of the outbreak. For 192 of the 290 patients initially recruited, a rapid test for non-structural protein 1 (NS1) antigen was available and performed at the hospital emergency unit. Blood samples from these 192 patients were then sent to the national laboratory in Maputo for further analysis. NS 1 was positive in 60 (31%) of the 192 tested patients and DENV IgM antibodies were found in 39/192 (20%). The CDC DENV-RT-PCR was then performed on 23 of the NS1 and/or IgM positive samples. The result showed 21/23 PCR positive, all for DENV-2 serotype. For three of the DENV-2 strains a sequence analysis was performed, showing genotype Cosmopolitan, the same strain that had previously been described from an outbreak in Tanzania, close to the northern border of Mozambique (173). An epidemic curve for the Mozambique outbreak showed that the highest incidence of DENV infection was seen between February and May, a period of heavy rainfall in Mozambique. With DENV-2 now established in Mozambique, the risk of resurgence and spread of the outbreak within rainy seasons is evident.

5.5 PAPER V

Unknown fever among travellers to the tropics- possibilities for early detection with PCR

In Paper V we analysed the material collected in the Swedish prospective multicentre study on tropical fever earlier described in Paper I, but with a different angle compared to the this publication. The primary aim now being to evaluate possibilities for early diagnosis with PCR, particularly in regard to patients with unknown fever. We thus analysed the acute samples with PCR. This differs from Paper I, where the focus was on serology, with a screening performed primarily on the convalescent serum samples, and in the event of positive titers further investigated by paired serum samples (acute and convalescent).

For this part of the study we identified 175 patients among the 415 totally included in the prospective study. These 175 subjects were selected on the basis of the following criteria:
- Patients from whom blood samples (serum and/or EDTA blood) only were available from the time of inclusion without a follow-up convalescent sera. These patients had not been included in the analysis performed for Paper I.
- Patients without a verified microbiological diagnosis, thus having tested negative in the serologic analysis of Paper I.
- To be eligible for PCR testing, the blood sample should have been collected within the first five days of disease.

The patient samples were analysed with PCR for the following arboviruses and bacteria: DENV, CHIKV, ZIKV, *Leptospira spp*., *Rickettsia spp*. The reason for not only studying arboviruses but also adding *Leptospira spp.* and *Rickettsia spp.* was our previous finding of additional Leptospira and Rickettsia cases in the serology study, making us interested in knowing if it would be possible to find even more cases with PCR:

From the originally include 175 patient samples, two were excluded from viral RNA analysis due to a negative internal control test for Beta-actin. In the remaining 173 samples we found that seven patients (4%) were DENV PCR positive. Four of those seven had not previously been tested for DENV infection, and could be considered as overlooked in the clinical diagnostic procedure. In the other three cases, the clinician had tested and found a positive IgM result for DENV in the acute sample. In spite of already having a probable diagnosis, neither a confirmation follow-up serum nor a previous negative serum sample showing seroconversion had been taken. In these three cases the new finding of a positive PCR for DENV confirmed the diagnosis (174). Serotyping with PCR was done for all seven PCR DENV positive cases, showing two cases of DENV-1, one case of DENV-2 and two cases of DENV-3. DENV serotyping with PCR was negative for two of the seven cases, which could be explained by the lower threshold for detection of the single probe universal RT-PCR used for DENV diagnostics in comparison to the in general higher threshold for the individual PCRs performed for serotyping DENV. Epidemiological data from the questionnaires showed that the DENV positive cases had visited Asia and South America, but not Africa. The DENV negative travellers had to a greater extent been to Africa. However, since there were only 7 individuals in the DENV positive group, it is difficult to draw any general conclusion regarding risk for exposure and destination of travel from those results.

PCR for CHIKV and ZIKV were all negative, corresponding to the material having been collected during 2005-2008, before these viruses had become more widely distributed in the tropical world. The spread of CHIKV started to escalate in 2006 with the outbreak on the islands in the Indian Ocean (175, 176). Regarding ZIKV, until 2007 it had only been found in Asia and Africa, when a local outbreak occurred in Micronesia without any recognised further spread until 2013 (61, 177).

The PCRs for *Rickettsia* and for *Leptospira* were all negative in the 172 patient samples included in this part of the study. Furthermore, when analysing 13 previously diagnosed cases of rickettsiosis, verified by paired serology and published in Paper 1, they were also negative in the PCR. Other studies screening febrile patients with PCR for rickettsia have also shown a negative result (178). Similar to our group of patients, they also had mild symptoms. The universal PCR method for rickettsia that our method is built on has been shown to be more sensitive than previous used PCR methods, and being able to detect rickettsial DNA in severe cases (105). Patients with rickettsiosis may have very few organisms present in blood during the acute phase, which limits the possibilities for genome detection. The presence of antibiotics diminishing the number of or eradicating the bacteria from the bloodstream can also explain the negative PCR result. Unfortunately, information about antibiotic treatment

given prior to sampling blood for the PCR analysis was not available. A review on Rickettsiosis noted that there were several new species identified as pathogens (101), which also could explain differences in serology and PCR results. This means that early laboratory verification of a rickettsial disease remains a challenge. A negative PCR does not exclude disease, while a positive PCR might indicate the need for prompt treatment. During this early phase the decision on antibiotic treatment thus still depend on the clinical suspicion of rickettsial disease (179). Serology, with an acute and a follow-up paired serum sample, could then be used to confirm the suspected diagnosis in a PCR negative patient.

When performing the PCRs for viral RNA we used Beta-actin as an internal control to verify the extraction procedure and the presence of RNA in the samples. Despite all samples having been handled and stored in the same way, 10 of the initial 175 patient serum samples were negative for Beta-actin. Furthermore, when the corresponding plasma for these 10 samples were tested, 8 of 10 became PCR positive for Beta-actin. All the samples had been collected during 2005-2008 and stored at -20°C. That means about 10 years of storage prior to the PCR procedures. RNases can degrade RNA samples, giving a negative PCR results. It might be that in some samples the RNA had degraded during the storage period. Other risks are inhibition, leading to a false negative result (180). In fact, no obvious explanation for the differences between plasma and serum has been identified neither when searching the Pub Med database nor when discussing with the helpdesk at the manufacturer of the extraction kits (181-184). According to the manufacturer (personal communication August 2017) the extraction method has been evaluated for plasma. The question is if RNA is more stable when stored in plasma rather than in serum for a long period of time. The question is also if Beta-actin as house-keeping gene varies in expression or is prone to degrade (185-187).

5.6 ETHICAL CONSIDERATIONS

Ethical approval for the Swedish multicentre study (Papers I and V) was given by the Regional Ethics Committee (Stockholm). The two studies performed in Mozambique (Papers III and IV) were approved by the National Bioethical Committee in Mozambique (Ministério da Saúde, Mozambique, comité nacional de bioética), with a corresponding approval from the Swedish Regional Ethics Committee (Stockholm) for the procedures in Sweden. Written consent was obtained from all the participants in the Swedish multicentre study (Papers I and V), and for the two studies where patients were included in Mozambique (Papers III and IV).

Paper II is a methodological development performed at the Public Health Agency of Sweden. The Department of Microbiology at the Agency is a reference laboratory and a Swedish referral laboratory for a number of pathogens causing infectious diseases originating from for instance tropical areas of the world. Methodological development is within the national authority's mission being stated and commissioned by the Government in the Regulation SFS 2013:1020, 3§, points 2 and 8 (https://www.riksdagen.se/sv/dokument-lagar/dokument/svensk-forfattningssamling/forordning-20131020-med-instruktion-for_sfs-2013-1020). While not requiring ethical permit nor written consent, in accordance with the allowance for the Public Health Agency of Sweden to use samples from the biobank of the Public Health

Agency of Sweden (former Swedish Institute for Infectious Disease Control, SMI) for such development and quality control relevant for the authority's mission, it goes without saying that both the researcher and the project leader are required to put ethical considerations high on the agenda. Routines, rules and regulation applying to laboratory work at the Public Health Agency of Sweden are to be strictly followed, including those relating to data protection.

In our studies, personal identifiers were anonymized at the including clinics before sending blood samples and questionnaires to be analysed. The key to personal data files was kept at the including centres. Similar regulations for sample collection and analysis also apply in Mozambique.

When it comes to positive research findings and potential impact on patient care, HIV was considered to be ethically disputable to include in the research panel of Study I and V where patients in Sweden were included. Decisions on testing for HIV were left for the clinicians to make in direct contact with the patients involved. In Study III and IV concerning patients in Maputo, where the endemicity for HIV is comparatively high, HIV testing as well as testing for malaria was routinely done by the clinicians on admission for fever. Thus the clinical routine testing differs between the two settings (Sweden-Mozambique).

All five papers are examples of the interaction between the individual patient perspective and the public health aspects. On one hand, our research focuses on individual patients when investigating the panorama of infections among febrile returning travellers or suburban population in Maputo. The aim is to contribute to a more correct diagnoses, better diagnostic tools and improved individual advice on preventive measures. On the other hand, we also wish to contribute the public health aspects on the national level, providing a basis for a broader preparedness against infections in Sweden or Mozambique. The results of these type of studies could contribute to decision making, while taking into account the importance of ethical considerations in the steps involving individual patients.

6 CONCLUDING REMARKS

REFLECTIONS AND PERSPECTIVES FOR THE FUTURE

The study approach in Paper I, III, IV, V is prospective descriptive. There are some differences between the studies, for instance in regard to age, gender and results of screening for malaria between Swedish travellers and Maputo residents with fever (see table 4). The risk for malaria is clearly higher for Maputo residents with 19% positive cases compared to the 7.5% malaria positive patients in the group of Swedish travellers. The difference in median age might on the other only reflect the difference in median age for the population as a whole. More women than men were included in the Maputo study. The opposite was seen in the Swedish study. It is interesting to note the differences in gender, but the limited information available makes it only possible to speculate on the factors behind the data and whether sampling effect or individual patient factors play a role.

Table 4. Swedish travellers and Maputo residents

	Paper I		Paper III	
	Sweden (travellers)		Maputo (residents)	
Number of patients	383		78	
Median age in years (age span)	37	(18-76)	29	(5-78)
Female gender	42 %	162/383	59 %	46/78
Malaria positive	7.5 %	29/383	19 %	15/78

Both travellers and residents in tropical countries face the risk of exposure to the infections we have studied in this thesis. When infected, the diseases will often present with unspecific symptoms. The individual risk of exposure can differ depending on factors not only associated with where in the tropics the person has been, but also such factors as type of housing, outdoor activities and risks taken. With communicable diseases emerging and re-emerging, increased travelling does not necessarily have to lead to an increased risk of exposure in general. Human interaction and exchange of knowledge and science, where all can contribute with different perspectives, is the source of better ideas for prevention. In this respect, both epidemiology and laboratory diagnostics can bring evidence to allow for better decisions on public health measures for both travellers and residents in tropical countries.

The diagnostic challenges related to unspecific symptoms and broad spectrum of diseases are common both regarding travellers and residents of tropical areas. On one hand we would wish for quick and easy ways to perform diagnostic tests with high sensitivity and specificity, if possible covering multiple diagnoses. On the other hand, in the perspective of travel and particularly to the tropics, adequate individual pre-travel advice and other preventive measures including relevant vaccinations should not to be forgotten.

Today, wrapping up the thesis, I would have liked to include a more recent follow-up study for the Swedish multicentre study on returning travellers. It would be very interesting to perform a PCR screening on acute samples for arboviruses, for instance including DENV, CHIKV, ZIK and maybe WNV. I would then have added sampling of nasopharyngeal secretion from the patients, to be able to perform a rapid test or PCR for influenza on this material (188, 189), PCR on serum samples not being a diagnostic choice for influenza. I would also consider the possibility of including HIV testing in the study analysis, taking into account the ethical implications of such a procedure.

Further arbovirus studies in Mozambique are needed, including a more in-depth analysis on the risk of human exposure to RVFV. Additionally, looking at influenza performing an all year round study with PCR on nasopharyngeal secretion could also be valuable.

IMPLICATION FOR POLICY; PRACTICE AND RESEARCH

This thesis wishes to contribute to the knowledge of the spectrum of infectious diseases brought to Sweden from the tropics by returning travellers. An extended analysis of blood samples from clinical cases with unknown or unspecified infectious aetiology has been made. This has been combined with a study of the viral zoonotic disease panorama among residents in a country in the tropics, namely Mozambique.

The knowledge obtained strengthens and adds to the information on which the current policies on diagnostics and prevention is based. The thesis concludes that additional laboratory screening with serology and PCR apart from routine clinical diagnostics can increase the number of cases with a verified microbial diagnosis. It could be emphasized not to neglect the following diagnoses: influenza virus infection (regardless of the time of year), dengue virus infection, leptospirosis and rickettsiosis.

While realizing the limitations of currently used laboratory methods for early detection of pathogens in endemic countries as well as among returning travellers, the thesis also points at the need for further development and research in this field.

LEARNING OUTCOMES

The most important learning outcome is for me not only the mere knowledge, but the insights and new ways of thinking I have reached through these studies. My critical thinking has been nourished and nurtured in meetings with teachers, fellow students of different ages and experiences and colleagues from other parts of the world. Bonding between our scientific fields through our common human interest gives me hope for the future of science.

The studies have provided me with an increased capacity to identify and formulate relevant research questions, both critically, independently and with scientific accuracy. My capacity to evaluate the work of others has also improved. Understanding the theoretical background and practical performance of molecular and immunological analysis necessary for the studies in the thesis was amazing. Achieving an increased awareness of strengths and weaknesses in different methods was also stimulating.

I am very grateful for having made this scientific journey. In times when facts without solid evidence sometimes become the generally accepted truth, understanding and searching for the evidence behind the facts is more than ever needed. The PhD education has been a real source of inspiration.

7 POPULÄRVETENSKAPLIG SAMMANFATTNING

Fler och fler resenärer återkommer från långväga resor, många har varit i tropiska länder och kommer hem med feber. Denna avhandling handlar om infektionssjukdomar med tropiskt ursprung. Två olika aspekter har studerats, dels effekten på invånare i ett tropiskt land, dels hur det ser ut bland hemvändande svenska resenärer som besökt tropiska länder.

I den svenska studien kunde vi konstatera att influensa är en betydligt vanligare orsak till feber efter resa än vad tidigare varit känt, hela 9% av feberfallen. Bland tropikresenärerna förekom fall av influensa oberoende av årstid, dvs. även utanför det vi vanligen kallar influensasäsong. Malaria är förvisso en viktig tropisk orsak till feber. I studien fokuserade vi dock framförallt på andra orsaker till feber än malaria, särskilt på sådana som inte fångats upp av vården vid sedvanliga utredningar. Vid en fördjupad analys fann vi bl.a. flera fall av infektioner orsakade av denguevirus, som också sprids via myggor i tropiska länder. Sammanlagt hade 4% av resenärerna en säkerställd infektion med denguevirus. Vi provade även att utröna om det gick att hitta ytterligare fall med hjälp av PCR, en nyare diagnosmetod som vi vidareutvecklat i syfte att täcka alla hittills kända varianter av denguevirus i världen. Det vi åstadkommit är en enkel och snabb undersökning som kan hjälpa kliniker att ställa en tidig diagnos av patienten. Förutsättningen är att ett blodprov tas inom fem dagar från insjuknandet. Med den metoden identifierades 7 fall i en grupp om 173 patienter med oklar feber efter tropikresa.

Mozambique är ett land där det fram tills nyligen endast har funnits ett fåtal vetenskapliga studier som beskriver läget rörande sjukdomar som t.ex. kan överföras med olika insekter. Vi genomförde två studier. I den ena undersökte vi patienter i en förort till Maputo. Resultaten visade att 19 % av patienterna hade haft en infektion med det myggburna viruset chikungunya. Ett antal av de undersökta patienterna, 13 %, hade antikroppar mot dengue-virusinfektion. I den andra studien tittade vi närmare på ett pågående utbrott av denguefeber i norra Mozambique. Vi konstaterade att dengue-virusinfektion av undergruppen serotyp 2 nu är etablerad i Mozambique. Det rör sig inte längre om enstaka fall utan om en sedan 2014 pågående förekomst. Undersökningarna i Mozambique gjordes i samarbete med lokala forskare och ansvariga på nationella myndigheter. Resultaten kan förhoppningsvis bidra till en ökad förståelse för sjukdomspanoramat i Mozambique och ingå i ställningstaganden till förebyggande åtgärder.

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