Seroepidemiology of vaccine-preventable and emerging RNA viruses in Rwanda

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To:
Kayihura Charles, your wife and all
your three kids; you died prematurely
My late Parents

My family

"Being deeply loved by someone gives you strength, while loving someone deeply gives you courage"
Lao Tzu

Abstract

Infectious diseases are a leading cause of death in sub-Saharan Africa, and in Rwanda diarrhea, lower respiratory and other common infections are linked to high mortality and morbidity. For children <5 years of age, neonatal/congenital disorders rank second among causes of death in Rwanda. However, neither the burden of, nor immunity to, fever-causing viruses in children and adults are currently known.

Despite recent progress of vaccination in Rwanda, childhood infections including measles are regularly reported to WHO. To assess immunity to vaccine-preventable viruses, and susceptibility to emerging arboviruses, we investigated the seroprevalence by ELISA of IgG to MeV, RuV, ZIKV, CHIKV, and WNV on samples from Rwandan and Swedish blood donors collected during 2015 for comparative studies.

The seroprevalence of MeV in Rwandan blood donors was low (71.5%) compared to that in Swedish donors (92.6%). This might be related to the previous one dose measles vaccine policy in Rwanda, (two doses were introduced in 2014). Yet, a comparably high seroprevalence was observed in older Rwandan and Swedish donors (90.4% versus 94.1%). The measles outbreak in Rwanda, 2010-2011, was investigated by PCR; sequencing revealed that these outbreak strains belonged to genotype B3, and were related to measles strains from neighbouring countries.

Rwandan blood donors were also tested for IgG to ZIKV and RuV, both viruses that can cause congenital infections. The ZIKV assay showed a seropositivity rate of 1.4%, and all 12 samples that were positive for anti-ZIKV IgG antibodies were negative by RT-PCR, arguing against active infection. Almost all women of childbearing age were found to be susceptible to ZIKV. In addition, a larger proportion of Rwandan women of childbearing age were seronegative for RuV (10.5%) compared to males (6.5%).

Among Rwandan donors, anti-CHIKV IgG and anti-WNV IgG antibodies were detected at the rates of 63% and 10.4%, respectively. The highest seroprevalence for both viruses was recorded within the Eastern Province,

with 86.7% and 33.3% for CHIKV and WNV IgG, respectively. Both *Culex* and *Aedes* mosquitoes were most prevalent in the Eastern Province. Swedish blood donors, as expected, showed a much lower seroprevalence for CHIKV, 8.5%. Surprisingly, the seroprevalence for WNV in Swedish donors was relatively high, 14.1%. This stimulated investigation for possible serological cross-reactivity with another flavivirus circulating in Sweden, i.e. TBEV. Dual seroreactivities of 78.6% and 70.3% were observed to WNV and TBEV in Swedish and in Rwandan donors, respectively. Furthermore, 19 of the 28 Swedish sera seropositive to WNV were confirmed by plaque reduction neutralization test as being anti-TBEV IgG antibody-positive, with possible cross-reactivity to WNV.

This dual seroreactivity to WNV and TBEV, seen in samples from both countries, was further characterized on pepscan analyses of E protein linear epitopes. Although we could define several novel IgG epitopes of both viruses, we found no explanation of their serological cross-reactivity. Instead, this phenomenon could be related to reactivity to discontinuous epitopes, or to IgG directed to flaviviral proteins other than the E protein. Surprisingly, the strongest peptide responses detected were from a pool of Rwandan plasma samples that reacted to linear epitopes of the E-protein of TBEV rather than WNV. This finding suggests the circulation of hitherto undiscovered tick-borne flaviviruses in Rwanda, which may share conserved epitopes with TBEV.

Keywords

Seroprevalence, measles virus, rubella virus, Zika virus, West Nile virus, chikungunya virus, tick-borne encephalitis virus, mosquitoes, linear epitopes, Rwanda, Sweden

Sammanfattning på svenska

Trots förbättrad profylax och behandling under senare år orsakar infektionssjukdomar fortfarande en betydande dödlighet i centralafrikanska länder inklusive Rwanda. Ett allmänt barnvaccinationsprogram med hög täckningsgrad har införts i landet, men grundläggande data gällande befolkningens immunitet mot viktiga infektionssjukdomar som mässling och röda hund saknas helt. Vidare har förekomst och spridning av myggburna virus som Zikavirus (ZIKV), Chikun-gunyavirus (CHKV) samt West Nilevirus (WNV) ej undersökts i Rwanda.

Målsättningen med avhandlingsarbetet har varit att kartlägga immunitet i form av IgG-reaktivitet (s.k. seroprevalens) mot dessa fem virus hos blodgivare från olika regioner inom Rwanda. Vad gäller mässling och röda hund kan arbetet ge underlag för riskbedömningar avseende utbrott av dessa två virus, och för mässling kan även effektiviteten av vaccinprogrammet, som startades 1982, bedömas. För mässling och röda hund har även diagnostiska data insamlats rörande akuta sjukdomsfall, samt för fosterskador efter infektion med röda hund under graviditet. Seroprevalensundersökningar på samma material av blodgivare har även genomförts mot tre kliniskt viktiga myggburna virus som samtliga ursprungligen upptäcktes inom Rwandas geografiska närområde: ZIKV, CHIKV och WNV.

I arbete I fann vi en betydligt lägre immunitet mot mässling, mätt som seroprevalens, hos blodgivare från Rwanda (71.5%) jämfört med svenska blodgivarkontroller (92.6%). Även antikroppsmängden, mätt som OD-värden hos de seropositiva individerna, var lägre hos Rwandiska blodgivare jämfört med de från Sverige. Trots den lägre graden av immunitet minskade utbrotten av mässling kraftigt under studieperioden, och nya fall inträffade framförallt i områden som gränsade till Burundi och Kongo-Kinshasa. Ett tydligt undantag från denna positiva trend var perioden under och efter folkmordet 1994, då vaccineringen nästan upphörde vilket ledde till omfattande mässlingsutbrott.

I arbete II fann vi en god immunitet mot röda hund, trots att allmän vaccination infördes först 2013. Det bör dock påpekas att >10% av kvinnliga blodgivare i fertil ålder i Rwanda befanns vara mottagliga för röda hund,

vilket understryker vikten av fortsatt vaccination. I samma arbete fann vi att i princip hela befolkningen var seronegativ för ZIKV, ett myggburet virus med fosterskadande potential. Fyndet betyder att gravida kvinnor skulle kunna drabbas av ZIKV-infektioner under graviditet, med risk för fosterskadande effekt, om detta virus skulle börja spridas i landet. Arbetet är en av få studier som undersökt immunitet mot ZIKV i Afrika, och resultaten stöder vikten av att en diagnostisk beredskap för detta virus införs i Rwanda.

Våra studier av immunitet mot myggburna virus fortsatte i arbete III, där förekomst av IgG mot CHIKV och WNV analyserades i samma blodgivarmaterial. Eftersom kommersiella tester av serologi mot CHIKV saknas, utvecklade vi en egen metod baserad på ett virusprotein som utvecklats som en vaccinkandidat. Antigenet fungerade väl med positiva och negativa kontrollsera, och vi kunde bestämma seroprevalensen för CHIKV till 63% hos rwandiska och 8.5% hos svenska donatorer. Seroprevalensen var allra högst i den östra provinsen, som också uppvisade störst förekomst av myggvektorn *Aedes*. Vid analys av IgG mot WNV fann vi, något förvånande, att seroprevalensen för detta virus var högre i Sverige (14%) än i Rwanda (10%). Eftersom fästingburet encefalitvirus, TBEV, ett relativt närbesläktat flavivirus, förekommer i Sverige men inte i Rwanda, analyserade vi IgG och även neutralisationsförmåga mot detta virus. Vi fann tecken på sannolik serologisk korsreaktivitet, som vi utredde närmare i Arbete IV.

Vi använde därvid en detaljerad metod (pepscan) för att kartlägga linjära IgG-epitoper hos glykoprotein E från WNV och TBEV. Vi kunde inte påvisa någon korsreagerande linjär epitop, trots likheter i aminosyresekvens mellan de två proteinerna. Istället fann vi flera specifika epitoper för bägge virus när vi undersökte poolade prover från Rwanda och Sverige. Ett intressant fynd var att den starkaste peptid-reaktiviteten påvisades hos rwandesiska prover gentemot TBEV. Fyndet kan tala för att hittills oupptäckta fästingburna flavivirus förekommer i Rwanda.

List of papers

This thesis is based on the following studies, referred to in the text by their Roman numerals:

I. **Seruyange E**., Gahutu J.B., Muvunyi M.C., Zena Uwimana G., Gatera M., Twagirumugabe T., Swaibu K., Karenzi B., Bergström T. Measles seroprevalence, outbreaks and vaccine coverage in Rwanda. Infectious Diseases 2016, 48:11-12, 800-807

II. **Seruyange E**, Gahutu JB, Muvunyi CM, Katare S, Ndahindwa V, Sibomana H, Nyamusore J, Rutagarama F, Hannoun C, Norder H, Bergström T. Seroprevalence of Zika virus and Rubella virus IgG among blood donors in Rwanda and in Sweden. J Med Virol. 2018; 90:1290–1296.

III. **Seruyange Eric**, Karl Ljungberg, Claude Mambo Muvunyi, Jean Bosco Gahutu, Swaibu Katare, José Nyamusore, Helene Norder, Peter Liljeström, Tomas Bergström. High seroprevalence of Chikungunya Virus IgG in Rwandan blood donors. Vector-Borne and Zoonotic Diseases, 2018. Under revision

IV. **Seruyange E**, Bergström T. Linear Epitope Analysis of Sera from Rwandan and Swedish Blood Donors with dual seroreactivity to West Nile and Tick-borne Encephalitis viruses. Preliminary manuscript, 2018

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Abbreviations

AIDS	Acquired immune deficiency syndrome
BCG	Bacillus Calmette-Guérin
CCHFV	Crimean-Congo hemorrhagic fever virus
CHIKV	Chikungunya virus
CNS	Central nervous system
CRS	Congenital rubella syndrome
CSF	Cerebrospinal fluid
DNA	Deoxyribonucleic acid
DRC	Democratic Republic of the Congo
DTP	Diphtheria, tetanus and pertussis
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
HepB	Hepatitis B
Hib	Haemophilus influenza type B
HIV	Human immunodeficiency virus
HPV	Human papilloma virus
IgG	Immunoglobulin G
IgM	Immunoglobulin M
MeV	Measles virus
ML	Microbiology department
MMR	Measles, mumps, and rubella
MR	Measles-Rubella
NPH	Nasopharyngeal
nsP	Nonstructural protein
nt	Nucleotide
OD	Optical density
ONNV	O'nyong-nyong virus
OPV	Oral polio vaccine
ORF	Open reading frame
PCV	Pneumococcal conjugate vaccine
PRNT	Plaque reduction neutralization test
RBC	Rwanda Biomedical Center
RCV	Rubella-containing vaccine
RNA	Ribonucleic acid
RPV	Rinderpest virus

RT-PCR	Reverse transcription polymerase chain reaction
RuV	Rubella virus
RVF	Rift Valley fever
SARS	Severe acute respiratory syndrome
SUH	Sahlgrenska University Hospital
TBEV	Tick-borne encephalitis virus
WHO	World Health Organization
WNV	West Nile virus
ZIKV	Zika virus

1 Introduction

1.1 Background

1.1.1 Infectious diseases in Africa

Infectious diseases are considered as a main health problem in Africa despite the important progress made in prevention, diagnostics and treatment during recent years. In sub-Saharan Africa, infectious diseases continue to be reported as the leading cause of death, and this mortality is dominated by gastroenteric and respiratory tract infections (Figure 1.1). Viruses cause most of these infections. In addition, vector-borne zoonotic infections caused by flaviviruses and bunyaviruses often show high prevalence in Africa.

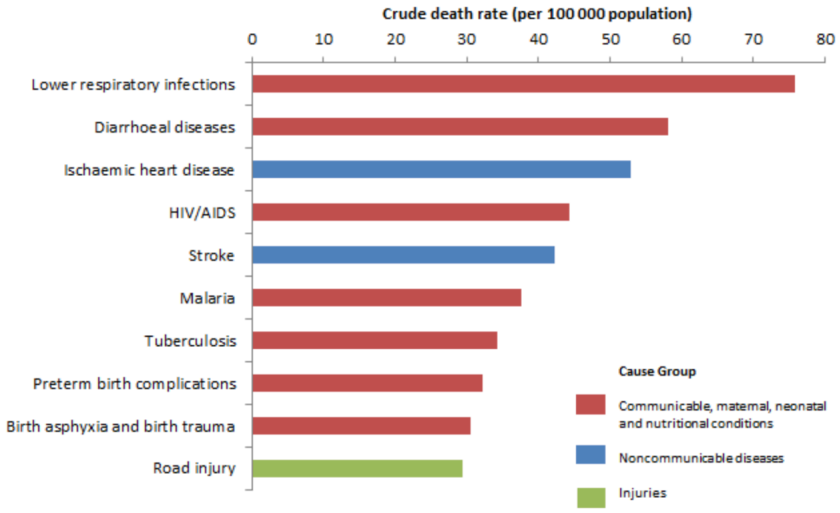

Figure 1.1. *Top 10 causes of deaths in low-income countries in 2016. Source: World Health Organization (WHO) 2018.*

As an illustration of the clinical importance of viral infections in Africa, the World Health Organization (WHO) reported 36.9 million cases of human immunodeficiency virus in 2017, where newly infected accounted for 1.8 million (5%). The same year, 940,000 deaths related to HIV were recorded worldwide. Further analysis of this mortality revealed that the WHO Africa Region was the most affected with around 70% of deaths, and this continent alone accounted for over two thirds of the total new HIV infections globally [1, 2].

Another example is the recent large outbreak of ebola in West Africa, which affected 28,000 where over 11,000 cases died. This demonstrated the lack of preparedness in Africa to respond to rapidly emerging health threats, as well as the fragility of existing health facilities [3].

Since the late 1800s, developed countries have alleviated the burden of infectious diseases by improving living conditions such as housing and access to clean water. Both these conditions are still poor in Africa. However, the introduction of vaccines during the 20th century, on a global scale, has lead to enhanced control of infections. This measure has rapidly been extended to low and middle-income countries, including Africa, with beneficial results [2].

1.1.2 Vaccination

Immunization, also known as vaccination, is one of the two most effective means to prevent infectious diseases, the other being improvements in sanitation and general living conditions. The discovery of immunity as a way to abrogate the occurrence of infectious diseases is a cornerstone of preventive medicine. Most important, infections are effectively hindered by active immunization where pathogens are introduced in form of vaccines. These can be made either from live attenuated infectious agents, or as inactivated, or detoxified agents or their subcomponents, which are administered to humans or animals in order to produce specific antibodies. Another, less efficient, way could be a passive immunization, for example injection of immunoglobulins, where exogenous antibodies are provided to ensure temporary protection against a targeted infection, as is the case with transplacental transfer of antibodies from the mother to the fetus [4].

Immunization started many centuries ago with variolation, the technique of inoculation of fluid from smallpox lesions into the skin of non-immune subjects. However, this practice showed severe side effects. Indeed, immunization did not

become a successful and more acceptable procedure until 1796 when Jenner inoculated fluid from cowpox (vaccinia) vesicular lesions into the skin of an exposed subject who mounted protection against smallpox [4]. This discovery was based on the observation by Jenner that milkers, who were exposed to cowpox in their work, were naturally immune to smallpox.

During the early 20th century, a rapid development of vaccines followed. As one example, Max Theiler discovered in 1930 that yellow fever virus can be attenuated by serial passage in mouse brain and chicken embryos, resulting in a successive reduction of its pathogenicity in monkeys. He also developed a test for measuring protective antibodies in mice [5]. Theiler's discoveries were essential for the production of the live virus vaccine against yellow fever that is still in use today, and he was awarded the Nobel Prize in medicine in 1951 for his work. Further progress was made by the implementation of cell cultures for vaccine development against a number of viruses such as polioviruses. Such vaccines could either be live attenuated or inactivated by formalin treatment.

The developed vaccines were then utilized in vaccination programs that gradually became implemented globally. Great results were achieved with the worldwide eradication of devastating diseases such as smallpox in humans in 1980, and of the measles-like rinderpest in cattle in 2011 [6]. The eradication through vaccination against other diseases such as polio, measles, rubella, and mumps is in progress [6-8]. Through a combination of vaccination programs and/or improved living conditions, the global incidence and burden of disease of many other infections have dramatically decreased during the last decades.

1.1.2.1 Vaccination in Africa

The preventive approach of controlling infectious diseases by vaccination was extended to Africa during the second half of the twentieth century [2]. From that period on, immunization coverage has gradually increased over the years. Diphtheria-Tetanus-Pertussis vaccination coverage increased considerably in Africa, from 57% in 2000 to 76% in 2015. As a result, vaccine-preventable diseases also decreased substantially, where for example deaths associated with measles were reduced by 86% between 2000 and 2014 [9].

Despite that progress, important disparities within and between countries due to social conflicts, health care system not functioning well, poverty or poor allocation of resources and inconvenient infrastructure is apparent (Figure 1.2). In

2016, Nigeria, Ethiopia, Democratic Republic of the Congo (DRC), Southern Soudan and Guinea were reported as the African countries with most under-immunized children [10, 11].

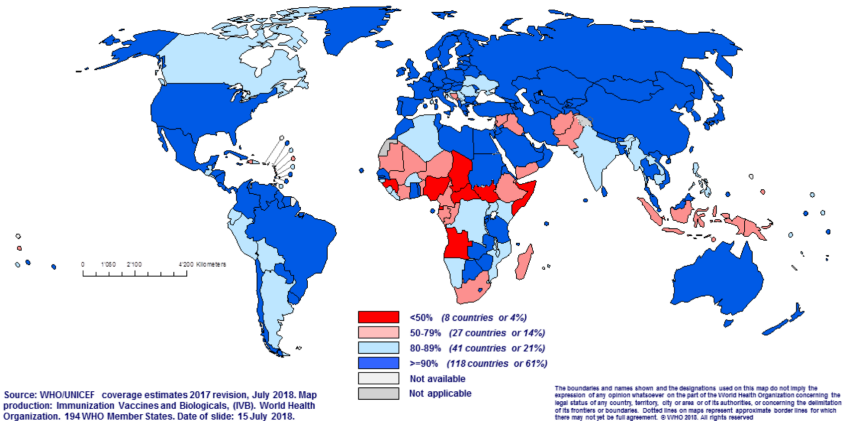

Figure 1.2. World: Immunization coverage with 1st dose of measles containing vaccines in infants, 2017. Most variations in vaccination coverage are recorded from the African continent where all the 8 countries, with vaccination coverage below 50%, are located. Source: WHO 2018.

1.1.2.2 Vaccination program in Rwanda

The vaccination program has been operational in Rwanda since 1980 and currently, immunization services are integrated into the routine activities of each health center. The program was implemented progressively (Table 1.1) with the introduction of the vaccines against tuberculosis (BCG), poliomyelitis (OPV), and tetanus for pregnant women, in 1980; diphtheria, neonatal tetanus, and pertussis (DTP) in 1981, later combined with hepatitis B and haemophilus influenza type B (DTP-HepB-Hib) in 2002; measles in 1981, also combined with rubella (MR) in 2013; streptococcus pneumoniae (PCV) in 2009; rotavirus in 2012; and human papilloma virus (HPV) in 2011 [12, 13].

Table 1.1 Rwandan immunization schedule

Vaccine	Total doses	Age and interval	Introduced in
BCG	1	Birth	1980
OPV	4	Birth, 6, 10, 14 weeks	1980
Tetanus (pregnant women)	2	During pregnancy	1980
DTP or DTP-HepB-Hib	3	6, 10, 14 weeks	1981 or 2002
Pneumococcal Conjugate Vaccine	3	6, 10, 14 weeks	2009
Rotavirus vaccine1	3	6, 10, 14 weeks	2012
Measles-rubella (MR) vaccine1	1	9 months	1981 then 2013
Measles-Rubella vaccine2	1	12 months	2014
HPV3	3	3 doses of HPV for each cohort of girls 9-14 yrs old	2011

Table 1.1 Immunization program in Rwanda including type of vaccines and their administration schedule, with the year of implementation [12].

In addition to those vaccines provided to the general population, yellow fever vaccine can be administered to anyone who seeks it, mainly to persons travelling to areas where risk of exposure to yellow fever infection is high. This is the only vaccine paid for by the recipient in Rwanda. Note that proof of yellow fever vaccination is mandatory for travellers coming into Rwanda from countries where there is risk of contracting this infection.

All vaccines are managed and only provided by the Extended Program of Immunization under the Rwanda Biomedical Center [14] and the Ministry of Health. There are no other in-country institutions or private companies providing vaccines.

The main outcomes of monitoring the immunization program efficacy are based on the vaccination coverage rate and incidence of cases reported. These criteria are used globally and are the only widely utilised in Africa. In addition, seroprevalence studies, which tests the level of IgG antibodies developed against viruses targeted by vaccines, can be a useful complement. In Rwanda, the reported vaccination coverage for the measles vaccine is > 95% [13].

1.1.3 Serosurveillance of infectious diseases

Seroepidemiology, the study of the prevalence of antibodies in serum or in other biological materials, serves as an important parameter to monitor the impact of infectious diseases in defined populations. To this end, direct assay of IgG antibodies as a measurement of the developed immunity is of paramount interest. Thus, seroepidemiology is an efficient means for the evaluation of the necessity of immunization to a given pathogen, and of the need of implementation of vaccination or other infection control programs [15].

In developed countries, serological surveillance has long been of importance for the formulation of national health policies, including the design of the vaccination programs. In England and Wales, serum samples were collected from 1986 to 1996, before the introduction of the MMR vaccine in 1988, and serological surveillance provided baseline data on immunity to these infections that directly influenced their national vaccination policy. To exemplify this, the 1994 measles and rubella campaign with the introduction of a second dose of MMR at 4 years of age was based on seroimmunity data. On the other hand, the decision not to implement a universal hepatitis B vaccination programme in this region was also founded on seroepidemiology reports [16, 17].

In addition to national serological surveillance programs within European countries, a European seroepidemiology network (ESEN) project, financed by the European Union, was established in 1996. This network co-ordinated and harmonised the serological surveillance of immunity to a number of vaccine-preventable infections and evaluated existing vaccination programs. Based on the recorded successes, they extended the project from 2001 to 2005 to investigate the seroepidemiology of several other infections and included new partner countries in southern and Eastern Europe [18, 19].

1.1.3.1 Antibody response and immunity to viral infections

The aims of viruses to survive and multiply require them to interact with the host cells and exploit their metabolism to their own benefit. Immune cells counteract the viral invasion and dissemination by innate or non-antigen specific and adaptive or antigen specific immune responses.

The innate immune response is stimulated by identification of viral constituents specific to different classes of pattern-recognition receptors (PRRs) such as the Toll-like receptors (TLR), retinoic acid inducible gene-I (RIG-I)-like recep-

tor (RLR), nucleotide oligomerization and binding domain (NOD)-like receptor (NLR). These receptors are important in initiating signaling for production of type I interferons (IFNs), proinflammatory cytokines and interleukin-1ß which play a role in clearing the infection, inducing apoptosis of infected cells and providing immunity to uninfected cells. Cytokines also stimulate recruitment of immune cells to the site of infection, thereby containing the infection followed by its clearance, but also aiding presentation of viral antigens for activation of the adaptive immune response [20, 21].

The adaptive immunity functions through T and B lymphocytes, which act in complementarity. Following the first exposure to the wild type virus or to a vaccine containing viral particles, the host responds directly through T lymphocytes where within 7-10 days of exposure, 50-60% of CD8 T cells are virus specific. Later, this response decreases progressively to remain with 1-10% of memory splenic CD8 viral specific cells. Thereafter, B lymphocytes response in form of IgG antibodies increases from 2 to 4 weeks of the infection and may last lifelong.

Following a secondary exposure to the same viral antigens, both T and B lymphocytes display antigen-specific memory where antibodies rise quickly in quantity and the peaks last longer than after primary challenge. Cytotoxic T cells also respond very quickly.

Neutralization of viruses by antibodies occurs mainly by IgG attachment to the viral site (the epitope) that could potentially interact with the cell-surface receptor of the host, and through lysis of the infected cell by complement. Other antibodies aggregate the infectious particles, thus reducing infectiosity of new cells.

The two main classes of antibodies used for serodiagnosis, IgM and IgG, behave differently with regards to the kinetics of the viral infection (Figure 1.3). The IgM appears early and mounts the initial antibody-mediated immune response by intercepting the virus and reducing virus binding to specific host receptors, and by aggregating the viral particles, thereby decreasing their infectivity. Later, and on secondary exposure, most IgM-producing cells switch to IgG, which is the major complement-binding and opsonizing antibody [22].

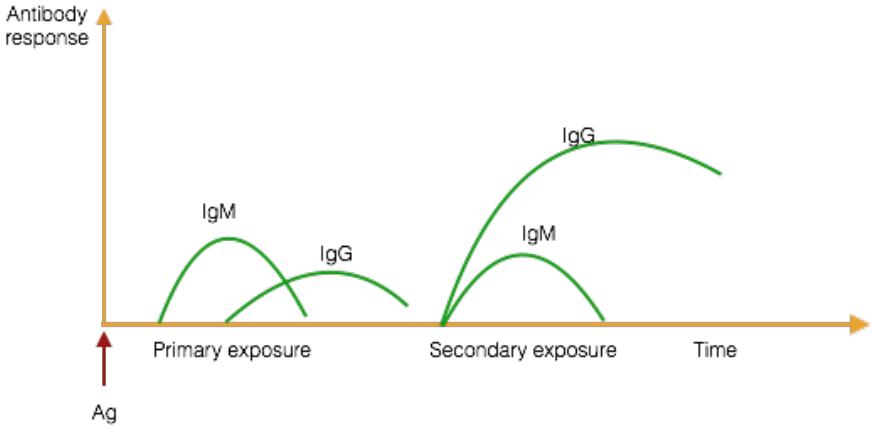

Figure 1.3 IgM and IgG antibodies response to primary and secondary exposure to a viral antigen.

1.1.4 Zoonotic diseases

Zoonotic diseases are naturally occurring animal infections that can be transmitted to humans. They differ in their etiologies, which can be viral (ebola fever, SARS, avian flu), bacterial (brucellosis, anthrax, tuberculosis), fungal (cryptococcosis, histoplasmosis) or parasitic (taeniasis, cryptosporidiosis). In addition, they manifest themselves with variable virulence; some occur with minor clinical signs such as sindbis virus while others, for example ebola virus, present severe complications with massive hemorrhage and dehydration leading to high mortality [23].

Persistence of zoonotic diseases in nature requires an appropriate animal reservoir (swine, sheep, camel, non-human primate, fox, rodents, bats, bovine, birds, etc) from where the pathogen might infect humans by direct contact with contaminated materials from the animal, or by an intermediate vector such as mosquitoes, ticks, flea, and flies [24, 25].

The ability of genetic adaptation through mutation influences the capacity of a given pathogen to fit within several different species, enabling the maintenance of the zoonotic life cycle including animals-vectors-humans. Several important zoonoses are of viral etiology and most commonly, such agents belong to the RNA group of viruses due to their frequent genetic variation resulting from a high RNA polymerase error rate and the lack of proofreading ability during their replication cycle [26-28]. Despite this fact, zoonotic RNA viruses can be remarkably stable under certain circumstances.

Though most zoonotic infections maintain the non-human to human life cycle, surprisingly, some evolve to become fully adapted to a transmission cycle exclusively within humans (eg: ebola, CCHFV, influenza A subtype H5N1, coronavirus, HIV, measles, etc) [29].

The global epidemiology of infectious diseases shows ongoing changes with arboviruses expanding to reach geographical areas where they were nonexistent before. This can often be influenced by alteration of the environment, such as by climate change favouring competent vectors. In addition, global trends in trade, travel and urbanization are factors resulting in enhancement of outbreaks of emerging zoonotic infections.

1.1.4.1 Mosquito-borne viruses in Africa

The high biodiversity found in Africa is also valid for important species of mosquitoes, which in turn have the potential to propagate many different viral pathogens. Such a rich bioenvironment might have constituted a strong network enabling the evolution of zoonotic infections. Over millions of years, most likely, viruses have adapted their life cycles to specific mosquito vectors and animal reservoirs. Later, these transmissions have also included humans because of their long coexistence with animals and vectors [30]. Among the most medically important mosquito-borne viruses reported from Africa, some are endemic and associated with outbreaks in humans such as yellow fever (YFV), dengue, or, in animals, Rift Valley fever. Many other viral infections such as Banzi, Bwamba, Bunyawera, Germiston, Ilesha, Lumbo, Middelburg, Ndumu, Ngari, Ntaya, O'nyong-nyong, Pongola, Semliki Forest, Shuni, Simbu, Sindbis, Spondweni and Wesselsbron were reported, often with nonsignificant clinical symptoms. However, benign viruses might suddenly surprise the global community, especially if introduced within an environment with immunologically naïve populations as recently occured with Zika virus in Brazil [31, 32], West Nile virus in

America [33] and chikungunya virus (CHIKV) in the Caribbean Islands [34, 35]. Interestingly, no serious outbreaks of these viruses have been reported recently from Africa although they all originate from this continent [36].

1.1.5 Geographical background of Rwanda

Rwanda is located in Central-East Africa, south of the equator between latitude 1°4' and 2°51'S, and longitude 28°63' and 30°54' E. It is a landlocked country with an area of 26,338 square kilometers, bordered by Uganda to the north, Burundi to the south, Tanzania to the east and the Democratic Republic of the Congo to the west.

The country is situated at high altitude with the lowest point at 950 meters and the highest at 4,507 meters above the sea level. Mountains are predominant within north, central and western regions while the eastern region is rich in savanna, swamps and plains.

The climate in Rwanda is sub-equatorial with an average yearly temperature of 18.5°C. This climate is also known to change from year to year, with extreme changes in rainfall that sometimes result in flooding or in drought [37]. Due to those unusual rainfalls with flooding, there is a rise in reproduction of mosquitoes that can vector diseases. The recent outbreak of RVF, reported since May 18, 2018 within four districts of the Eastern Province, was believed to be linked to the heavy rains and floods that hit Rwanda during March and May, 2018 [38].

1.1.6 Mosquito-borne diseases in Rwanda

Despite having an ecological environment (most of the country being under 2,300 m above sea altitude) suitable for mosquitoes [39], and being a sub-Saharan African country where most arboviruses originate, little is known about mosquito-borne diseases in Rwanda. One exception is malaria, which is a parasitic disease that has been controlled through a national program within RBC and the Ministry of Health. In this context, the current outbreak of RVF might be a warning sign of other possible mosquito-borne viral infections which have not been controlled for, especially those sharing the same vector as RVF [38]. We contributed within this field by assessing the seroprevalence, from blood donors, to CHIKV, West Nile virus and Zika virus that are transmitted by the two mosquito species *Culex* and *Aedes* that also vector RVF.

In addition, we explored serologically, for the first time in Rwanda, protective response from immunization to measles and to rubella viruses among blood donors.

1.2 Measles virus

1.2.1 Historic aspects of measles

The first measles-like clinical characteristics were reported as early as the 9th century when a Persian physician Abu Becr (known as Rhazes) differentiated measles from smallpox. However, measles might have been described already in the 6th century as a modification of smallpox, under the name of *hasbah* (eruption) in arabic [40].

Epidemics of rash-like diseases have been reported from Europe and the Far East since ancient times. It is hypothesized that measles may originate from the Middle East, which in those times had sufficient population density for the maintainance of transmission cycles of measles. It took until the 11th and 12th centuries before those epidemics of what could have been measles infections were identified as a childhood disease, in 1224 [41].

In differentiating those lesions from plague, the Europeans gave them the name of "morbilli" from the Italian meaning "little disease". Later, in 1763, the disease was named as measles after Sanvages who called it rubeola from the Spanish [40].

High morbidity and mortality associated with measles was observed among non-immunized populations. From the measles epidemic that occurred in the Faroe Island in 1846, the Danish physician Peter Panum described this disease as an airborne, highly contagious infection, with an incubation period of 14 days, but conferring lifelong immunity. A mortality rate of 26% associated with measles was reported on the Fiji Island in 1875. The introduction of Old World diseases, especially of measles and smallpox, through European exploration of the New World, into naïve native Amerindian communities was associated with at least 56 million deaths [40, 42].

1.2.2 Virology: genes, proteins and replication

Measles virus (MeV) is a spherical, enveloped virus containing nonsegmented, single-stranded, negative-sense RNA. This large (100-300 nm) pleomorphic virus belongs to the *Morbillivirus* genus of the *Paramyxoviridea* family.

It is closely related to the bovine rinderpest virus (RPV), and MeV might very well originally have emerged from that virus. The bovine-to-human transfer might have occurred as a consequence of livestock farming, with cattle and humans living in a close environment [43].

The analysis of genes encoding the envelope proteins N and H showed that the Time to the Most Recent Common Ancestor (TMRCA) of the currently circulating MeV strains was 1921 and 1916, respectively, for these two genes. Of interest in this context, the date of divergence between MeV and RPV was estimated to have taken place already during the 12th century [41]. It can be noted here that in 2012, after a long and tedious campaign including veterinarians, RPV was the 2nd virus ever to be eradicated from our planet [44].

The MeV genomic RNA has a molecular weight of 4.5 kDa, containing 16,000 nucleotides (nt) and six genes encoding six major structural proteins: the membrane envelope (hemagglutinin H and fusion F proteins), the matrix M protein, and the three proteins belonging to the nucleocapsid complex, nucleoprotein N, phosphoprotein P and large protein L (Figure 2.1).

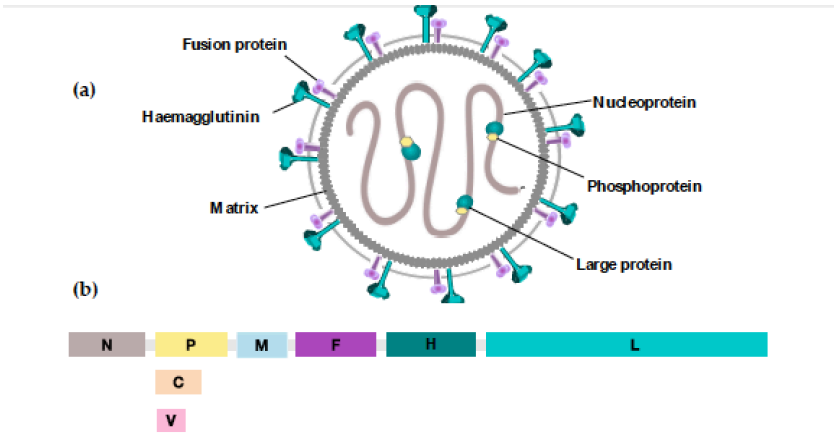

Figure 2.1 Schematic representation of (a) MeV proteins and their location on the virus particle and (b) the linear MeV genome [45].

Entry of MeV particles into the host cells is initiated by the H protein, which facilitates attachment to the cell surface, via CD150/SLAM and nectin-4/PVRL4 host receptors for wild-type virus, or CD46 receptors for the vaccine strains, followed by fusion mediated by the F protein. Post entry, the matrix M protein facilitates interaction between envelope proteins and the nucleocapsid for maturation of virions. The nucleocapsid N protein surrounds the genetic material, the viral RNA. In addition, the P and L proteins bind to the RNA and serve in transcription and replication. The two additional nonstructural proteins C and V (both encoded from the P gene) most probably also play a role in the regulation of transcription and replication of the virus [46-48].

The viral replication pattern often determines the degree of acquired mutations. Viral mutant strains, also called genotypes, occur according to the amount of viral replication. The more the type of virus replicates, the more diverse it becomes; therefore, the transformation into new genotypes by genetic drift increases with multiplicity. Escape mutants within immunodominant viral genes may induce new viral serotypes, which can be resistant to vaccines or be responsible for spread within hitherto immune populations. To this end, it is vital to ensure a close and frequent monitoring of genotype emergence, especially from pathogens targeted by vaccines. Such preparedness includes implementation of preventive measures against unexpected massive outbreaks, as was exemplified by recent outbreaks of ZIKV and Ebola virus.

In addition to antigenic drift, viruses may evolve through antigenic shift, as is the case for influenza virus type A. This virus has a yearly mutation rate of 1x 10^{-3} to 8x10^{-3} amino acid substitutions per site in addition to the 256 (2^8) possible combinations of the eight gene segments from reassortment between different types of influenza virus, with the potential for switching to a new serotype almost yearly [49, 50]. This is the reason why the influenza vaccine is often renewed yearly to respond to the circulating serotype of a certain season. Due to the practice of yearly influenza vaccination, it has been observed that the IgG antibody quantities against influenza virus antigens are much higher, 50x, 10x and 5x respectively, as compared to those directed against mumps, measles, and rubella viruses. The presence of high levels of anti-influenza virus IgG might interfere with the clearance of antigens from the newly administered vaccine, therefore reducing or inhibiting its protective effect [51]. Determination of the optimal timing for vaccination of infants to avoid interference with maternal antibodies could influence the vaccination pattern of highly mutant viruses. Some vaccines fail to protect seronegative individuals or, even worse, other vaccines may increase the severity of the infection as observed with dengue virus vaccines [52-54]. With regards to these pitfalls in vaccination, but also to the

dynamics of viral genetics, regular surveillance of MeV genotypes, which present with ever expanding clades (Figure 2.2), is essential. Surveillance studies must also focus on the differences reported in neutralization testing of the various genotypes [55].

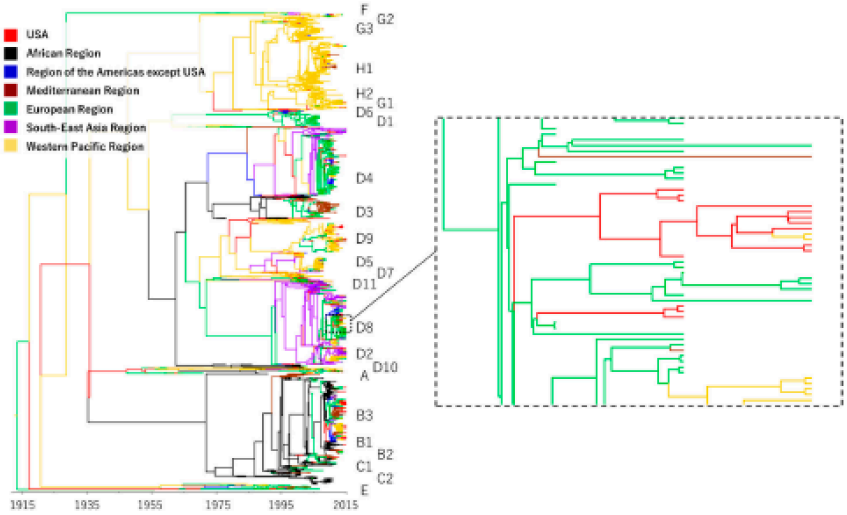

Figure 2.2 Phylogenetic tree of MeV, with branches colored according to the geographic origin of the different strains [56].

Genotyping studies of MeV are often based on nucleotide sequence analyses of the H and/or N genes. Nucleotide variability within those genes is low and is estimated at 8% from sequences of all strains analysed. The most variable site was found to be the 450-nucleotide region, with 12% variability between wild type MeV, which encodes the COOH-terminus of the N protein. Records of the global distribution of MeV, updated in 2015 by WHO, revealed eight clades of MeV, namely A, B, C, D, E, F, G and H. Subsequently, twenty-four genotypes, designated A, B1, B2, B3, C1, C2, D1, D2, D3, D4, D5, D6, D7, D8, D9, D10, D11, E, F, G1, G2, G3, H1, H2 were identified. However, 18 of these genotypes were last detected until 2011 and only six genotypes are reported thereafter. The currently circulating MeV genotype H1 was found in Western Pacific, Eastern Mediterranean Europe, Southeast Asia and America regions. The B3 genotype was endemic in sub-Saharan Africa before 2014, but has since been found to be distributed worldwide where it was associated with outbreaks within 49 countries, the largest one being in the Philippines. The genotype D8 was reported

globally in 2014 but previously it was only found in southeastern Asia, particulary in India. Genotype D4 was reported from Southeastern Asia, Western Pacific, Eastern Mediterranean region, Europe, America and Southern Africa. The D9 genotype was identified in Europe, Southeastern Asia and Western Pacific regions while G3 was reported in Southeastern Asia and Western Pacific regions [57-62]. Some of those genotypes were linked to endemic transmission and others to imported cases.

Despite the existence of all those various genotypes, MeV presents with only one serotype due to the similarity of surface antigens across all MeV strains. This permits the potential neutralization of all measles genotype strains by serum samples from vaccinees or subjects infected with measles, although neutralizing titers can vary for the different genotypes [63]. One conserved region is the core part of the nucleocapsid protein (Ncore), which is immunodominant as regards antibody response [64]. However the protective role of these antibodies is not fully known.

As mentioned above, the replication of MeV starts by the attachment of the H protein to host cell surface receptors (CD150/SLAM, CD46 and nectin-4/PVRL4), which activate conformational changes in the F protein, facilitating fusion of the viral envelope with the plasma membrane, and the release of the ribonucleoprotein complex into the cytoplasm. The viral RNA then serves as template for the RNA-dependant RNA polymerase (encoded by L gene) and is transcribed directly into viral mRNA; there is no DNA intermediate. H and F proteins facilitate the fusion of infected cells with neighbouring cells resulting in the formation of multinucleated giant cells (syncytia), which are a hallmark of MeV infection [48, 65, 66].

1.2.3 Transmission, epidemiology and clinical presentation

MeV only infects humans and non-human primates. It is highly contagious and the infection takes place through inhalation of viral particles produced mainly by coughing or sneezing. In naïve populations, a single measles case might infect more than 90% of his/her close contacts to cause severe large outbreaks with high morbidity and mortality. The peak of MeV replication takes place within the respiratory tract, and the most contagious period, occurs 4 days before the onset of clinical signs and continues until 4 days after the appearance of rash. This makes it difficult to isolate the index case and stop the spread of the infection within the community. Transmission of MeV within physicians' offices,

waiting areas in hospitals, sport complexes, and congregation at water collection sites have been reported [67, 68]. However, MeV confers a lifelong immunity and consequently remains a childhood disease within highly endemic areas.

There is no reservoir host apart from humans. Thus, to maintain persistence of MeV and continuous transmission, a large human population ranging between 250,000 and 500,000 at least is required [41, 69-71]. This is the reason why small and bottleneck populations often are non-immune to measles. As one example, the native populations of the Americas were highly susceptible to measles during the Spanish invasion from the 16th century an onwards.

Before the introduction of the live attenuated MeV vaccine in 1963, measles was associated with a high mortality among children. In the pre-vaccination era, there were around 135 million cases with 7-8 million of deaths yearly, worldwide. Following improvement of socioeconomic conditions with rich nutrition and access to well-equipped health facilities, cases of measles-related death decreased. The introduction of the measles vaccine in 1963 was associated with the control of the disease. Unfortunately, a rise of measles-related mortality and morbidity is presently recorded, especially among children, as a consequence of low immunization coverage due to a vociferous anti-vaccination movement in Europe. In addition, war, poor socioeconomic status, lack of appropriate health infrastructure, and poor management of natural disasters in developing countries, have also exacerbated the problem of low immunization coverage [72-74].

Recently, over 41,000 measles cases were identified in the European region from January to June 2018. This number, recorded during only a half year, far exceeds total cases reported yearly for the last 8 years in Europe. The highest and lowest number of cases reported from 2010 to 2017 were 23,927 and 5,273 for the years 2017 and 2016, respectively [75]. Globally, the number of clinically confirmed cases of measles reported between January and August 2018, exceed the total number recorded for 2017 (83,951 vs 75,552) confirming the decline of control measures within the WHO region [76].

Clinical characteristics of measles infection appear around 10 to 14 days after infection. Symptoms start with mild to moderate fever, persistent cough, runny nose, conjunctivitis and sore throat followed by high fever. The rash, which appears first on the face, spreads down to the body, and lastly to the extremities, including palms and soles. Koplick spots, a rash present on mucous membranes of the mouth, are considered pathognomonic for measles (Figure 2.3). It occurs from 1-2 days before the rash, to 1-2 days after the rash.

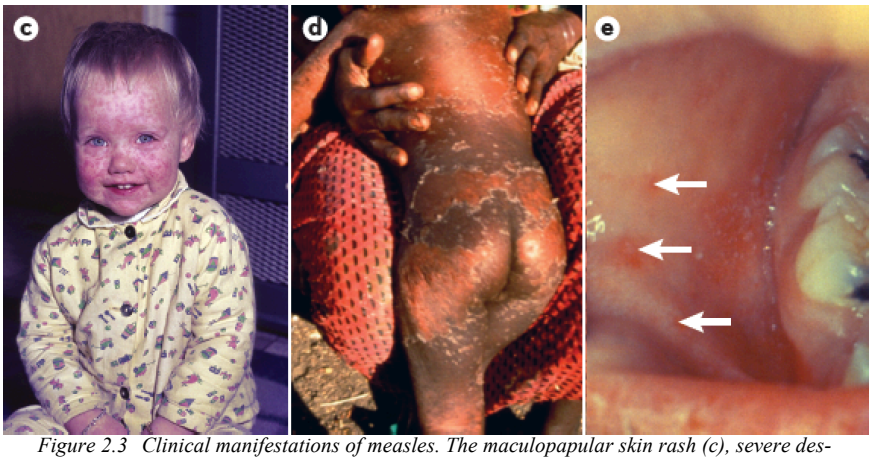

Figure 2.3 Clinical manifestations of measles. The maculopapular skin rash (c), severe desquamation of maculopapular skin rash (d) and the Koplik spot (e; white arrows). Reproduced with permission from Springer Nature [66].

Recovery from the disease might be spontaneous with measles rash receding gradually, but the infection might also be associated with severe complications, which could result in death. Among such complications are otitis media, bronchitis, laryngitis (croup), and pneumonia, which often is fatal among immunocompromised patients. In addition, MeV is a neurotropic virus and encephalitis may occur directly after the infection or later, after many months or even years after the acute stage. Among pregnant women, measles infection may cause preterm labor, low birth weight or maternal death. Another rare complication, the subacute sclerosing panencephalitis (SSPE), a degenerative central nervous system disease, is reported to be due to the persistence of measles virus infection within the brain. Onset occurs on an average 7 years after measles (range 1 month-27 years) [68, 77, 78]

1.2.4 Diagnosis

The clinical diagnosis of measles is often confirmed by the detection of IgM response, which may persist up to 1-2 months after the onset of rash. Up to 20% of IgM results may be falsely negative if samples are collected within the first 72 hours of appearance of rash. IgG antibodies to measles appear later, and measles

infection can be confirmed by comparing two samples collected at different time points, where the first sample is collected directly after the onset of the rash and the second taken 10-30 days later. Both samples should be analysed together ie. in parallel, by same test. Acute measles is confirmed if the second sample shows a four-fold rise in MeV antibodies titer as compared with the first sample.

Modern techniques of molecular biology also contribute to the diagnosis by demonstrating measles RNA, for example by RT-PCR, in body fluids such as blood, urine, nasopharyngeal aspirates, or throat swabs. Isolation of MeV can be performed from the same sample materials. This method is not recommended for routine diagnosis. However, isolation followed by genetic sequencing plays an important role in the molecular epidemiologic surveillance of the measles strains circulating in the community, and in determining the possible origin of the measles outbreak [43].

1.2.5 Treatment and prevention

There are no antiviral drugs available against MeV. Infected patients are treated with supportive therapy, which may include antipyretics and fluids. Appropriate antibiotics are considered for bacterial superinfections. Vitamin A is also used to decrease the severity of measles, especially among patients with vitamin A deficency or the malnourished. However, this vitamin was reported to reduce seroconversion in vaccinees, and therefore is not recommended for administration during or directly after vaccination [68].

The overall most effective prevention against measles is the administration of the live attenuated vaccine that confers a persistent immunity to measles. Fever and rash may be manifested in about 5-15% of children 7 days after vaccination. Passive immunization, with immunoglobulin, against measles is sometimes recommended. This should be given within 6 days of an exposure to abrogate infection, especially to children on chemotherapy, on radiotherapy or patients immunosuppressed by HIV [68].

1.3 Rubella virus

1.3.1 Virology: genes, proteins and replication

Rubella virus (RuV) is a single-stranded, nonsegmented, positive-sense RNA virus and the only member of the genus *Rubivirus* belonging to the *Togaviridae* family. RuV is also the only known togavirus not transmitted by a vector.

De Bergen, a German physician, first described infection from RuV in the early 1800s. He called it Rõtheln, later known as *German measles* because it caused a milder form of exanthema compared to measles. In 1866, H. Veale proposed the name Rubella [79].

Rubella viral particles (virions) have variable morphology, where most are spherical and measure between 57-86 nm of diameter [80]. The viral genome contains two open reading frames (ORFs) where the short one of 3,189 nucleotides encodes three structural polypeptides: two glycoproteins, the E1 and E2, which spike from the lipid membrane envelope; and the capsid protein C containing proline and arginine residues, which bind to the viral RNA to form the nucleocapsid. The long RuV ORF of 6,345 nucleotides encodes two nonstructural polypeptides, the p150 and p90, which are important during viral replication.

RuV has only one serotype. The glycoprotein E1 was found to be the most dominant antigen and initiates the binding of the virus to the host cell receptor. It also facilitates membrane fusion in presence of low pH and calcium ions. E2 glycoprotein binds the capsid protein to the membrane and is responsible for the folding and transportation of E1 through cellular compartments.

Additionally, three N-linked glycosylation sites were found to be located on the E1 protein of all RuV strains, and these glycans contribute to adequate folding of E1 to exhibit suitable antigenic and immunogenic epitopes. In contrast, the E2 protein has N-linked glycosylation sites that are strain dependant.

Furthermore, six nonoverlapping epitopes were found on the E1 protein, by the use of monoclonal antibodies. Some of these epitopes were associated with hemagglutination and neutralization. However, the E2 protein, found to be disulfide-linked to E1 in mature virion, is not well exposed and therefore less antigenic [79, 81-84].

Penetration of RuV into the host cell takes place through endocytosis. The viral capsid protein releases the RuV RNA, into the cytoplasm of the host cell, by its conformation change from hydrophilic to hydrophobic that occurs at pH 5.0 to 5.5.

Within the infected host cell, the two viral RNA species play an important role during the replication (Figure 3.1). The 40S RuV genomic RNA acts as a messenger for the nonstructural proteins, but also as a template for the synthesis of its complement, the 40S negative-sense RNA strand. This RNA later serves as a template for the transcription of 40S RNA and 24S RNA. Newly formed 40S RNA is linked to the RuV capsid protein to form nucleocapsids. The 24S subgenomic RNA is translated into structural proteins [84].

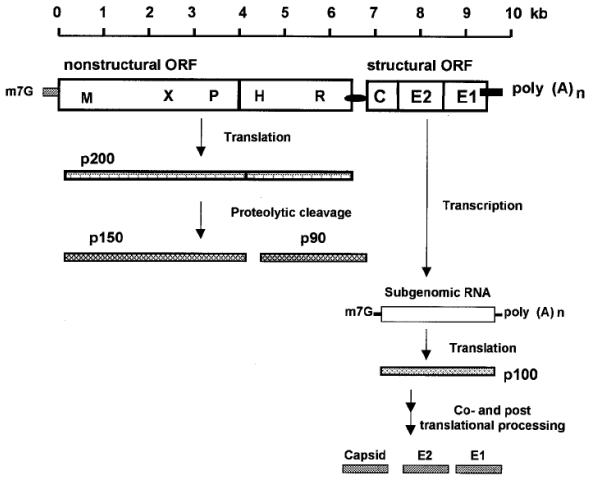

Figure 3.1 Schematic representation of the translation and processing strategy of the RV non-structural and structural proteins. The RV genome comprises two long nonoverlapping ORFs, with the 5' ORF coding for the ns proteins and the 3' ORF coding for the structural proteins. A polyprotein precursor, p200, is translated from the 5' ORF of the RV genomic RNA and undergoes cis cleavage to produce two non-structural proteins, p150 and p90. The locations of the putative amino acid motifs for methyltransferase (M), X motif, papain-like cysteine protease (P), helicase (H), and replicase (R) are indicated on the 5' ORF. The RV structural proteins are synthesized from a 24S subgenomic RNA transcribed from the 3' ORF. A polyprotein precursor, p100, is translated from the subgenomic RNA and undergoes several posttranslational modifications to ultimately produce the mature capsid (C), E2, and E1. Reproduced with permission granted from the American Society for Microbiology [84].

1.3.2 Transmission, epidemiology and clinical presentation

Humans are the only known host for RuV. Infected patients may transmit the virus, from 10 days before and 15 days after the eruption of the rash, by shedding droplets of respiratory secretions produced by coughing. Also, secretions from children with congenital rubella syndrome (CRS) may transmit infection during the neonatal period to susceptible individuals [85, 86]. Infection occurs only once, since RuV immunity is lifelong.

Rubella was not considered as an important disease until 1941, when Gregg linked it to congenital malformations (see section 1.3.3). This infection was also known as the *third disease* in reference to other more important exanthematous diseases such as measles and scarlet fever.

Before the introduction of the vaccine, rubella was reported to be more predominant in children of 5 to 9 years old with minor outbreaks taking place every 6 to 9 years. Important outbreaks were occurring at intervals of up to 30 years.

However, the introduction of the vaccine in 1969 modified the course of epidemics where no new large outbreaks presently occurs, and minor outbreaks are limited to communities where the index case is in close contact with susceptible individuals in schools, military camps, etc [86, 87].

The WHO highlights the important reduction of rubella cases globally due to the increased vaccine coverage. In 2000, there were 670,894 rubella cases. This number decreased to 22,361 rubella cases in 2016, while the number of countries that introduced immunization with rubella-containing vaccine (RCV) increased from 102 in 2000, to 165 in 2016. Interestingly, the WHO region of the Americas, and 33 of the 53 countries of the European region have successfully eradicated rubella as of the 2016 report [88]. Other regions might also be able to eliminate rubella if the herd immunity could be maintained between 85% and 91% [89].

RuV is divided genetically into two clades, 1 and 2, based on an 8-10% difference at nucleotide level from a sequence of 739 nucleotides, (nt positions 8,731 to 9,469) of the E1 protein coding region, as recommended by WHO. There are hitherto 13 reported genotypes where 10 (1A-1J) belong to clade 1 and the other 3 (2A-2C) to clade 2. Since 2010, only 4 genotypes are circulating with 2B being the most widely distributed virus strain, followed by 1E, while 1G and 1J are less frequently detected and more locally distributed. RuV strains of 1960, including viral strains contained in the vaccines, were of genotype 1A. Its occa-

sional detection might be a result of laboratory contamination since this strain is frequently used in the laboratory [90, 91].

Genotype 2B was reported globally whereas the genotype 1E was mainly found in eastern Asia and genotype 1G mainly in Africa. The rare findings of genotypes 1G and 1J might correspond to the under surveillance of rubella within countries where they might be preponderant (Figure 3.2).

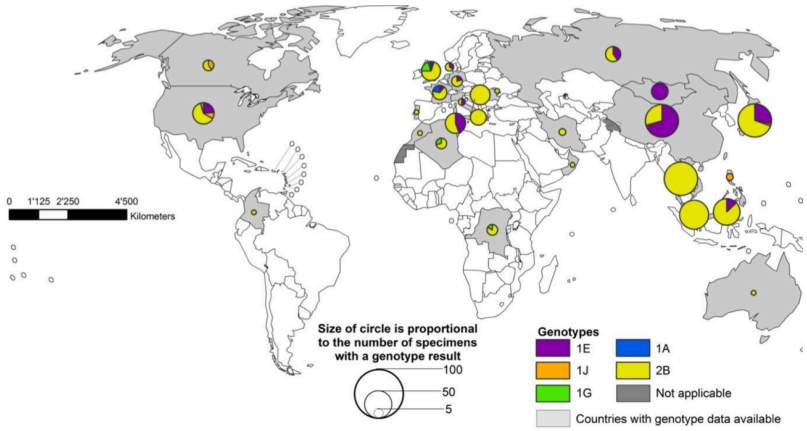

Figure 3.2 Global Distribution of Rubella genotypes: 2010-2015. Source: WHO

Rubella is often asymptomatic, but may manifest itself with mild symptoms such as fever, fatigue, and rash that starts on the face and disseminates over the whole body. Other signs are joint pain (a frequent symptom in adults), sore throat, and lymphadenopathies. Symptoms are more frequent and severe among female patients compared to males [84, 87, 92]. Rubella infection is associated with complications such as arthritis, encephalitis, hemorrhagic manifestations, and rarely orchitis, neuritis and the late syndrome of progressive panencephalitis. These complications are most severe in the fetus and fetal infection is associated with a high risk of severe congenital anomalies [93].

1.3.3 Congenital rubella syndrome

This is a complication, which occurs in the fetus if RuV infects a pregnant woman during pregnancy. Infection during the first trimester of pregnancy is associated with the highest risk for the fetus to develop severe malformations [86]. In fact, the fetus has a 65%-85% risk of being affected by multiple con-

genital malformations and/or spontaneous abortion if the infection occurs during the first 2 months of pregnancy. During the third month, the risk decreases to 30%-35% and CRS might manifest itself with only one malformation such as heart defect or deafness. Infection during the fourth month is associated with 10% risk of developing CRS, which then often is shown as deafness alone.

CRS is linked to the accumulation of necrotic tissues, from chorionic epithelium and endothelial cells, within the fetal circulation and organs. In turn, this leads to disturbance of mitosis and of development of precursor cells by inhibition of intracellular actin assembly; but the pathogenesis also involves upregulated cytokines and interferon [89].

Common manifestations of CRS are low birth weight and decreased head circumference, respiratory distress secondary to cardiac anomalies, cataract, hearing impairment, splenomegaly, jaundice, thrombocytopenic purpura, depressed neonatal reflexes, developmental delay, and meningoencephalitis [85, 87, 92].

1.3.4 Diagnosis

Rubella is confirmed by detection of IgM antibodies from serum or oral fluids, or of viral RNA isolated from nasopharyngeal secretions, oral fluids, urines or cataract tissue. A four-fold increase of rubella-specific IgG antibodies from serum samples collected during acute or convalescent phase of the disease, or the amplification of rubella virus RNA by RT-PCR confirms the diagnosis.

Important consideration should be given to the time of sample collection. Rubella-specific IgM positive results are often reported from most of the samples collected at the eruption of rash until 5 days after, while isolation and/or RNA detection of the virus is possible from the onset of the rash until at least 10 days postinfection. However, in the case of CRS, IgM specific to RuV, as well as viral RNA, may persist for months and thus be readily detected from samples collected at a later period of the disease [89].

1.3.5 Treatment and Prevention

There is no antiviral treatment against RuV. Its management is based on treatment of symptoms.

Prevention is done by administration of a live attenuated rubella virus vaccine, often given in combination (as RCV) with measles and mumps known as MMR vaccine. RCV is contraindicated in pregnant women and in immunosuppressed individuals, especially those with active HIV/AIDS [94]. Rubella-specific IgG antibodies may test as false positive after infection with parvovirus or Epstein-Barr virus, or due to presence of Rh factor [89].

1.4 Chikungunya virus

1.4.1 Virology: genes, proteins and replication

Chikungunya virus is a mosquito-borne RNA virus that was isolated and reported for the first time by Marion C. Robinson and W. H. R. Lumsden during an outbreak that occurred in Newala District of Tanzania in 1952 [95-97].

This virus is a member of the *Togaviridae* family comprising the two genera *Rubivirus* of RuV species and *Alphavirus* to which, in addition to CHIKV, many other viral species belong. Among these are Sindbis virus, Semliki Forest virus, O'nyong-nyong virus (ONNV), Venezuelan Equine Encephalitis virus, Eastern Equine Encephalitis virus, Western Equine Encephalitis virus, Ross River virus, and several others.

Alphaviruses including CHIKV have comparable structures and replication cycle processes. CHIKV is an enveloped, single-stranded, positive-sense RNA virus. Its genome contains around 12,000 nucleotides that encode four nonstructural proteins (nsP1, nsP2, nsP3, nsP4) that are translated from the 5'two-thirds of the genomic RNA. In addition, five structural proteins [98] C, E3, E2, 6K, and E1, are translated from the 3' one-third of the genomic RNA known as 26S subgenomic, positive-sense RNA. This RNA is later transcribed from the negative-stranded intermediate RNA during the replication cycle of the virus. Thus, CHIKV genome is arranged as follows (Figure 4.1) 5'cap-nsP1-nsP2-nsP3-nsP4-(junction region)-C-E3-E2-6K-E1-poly(A) 3' [99, 100].

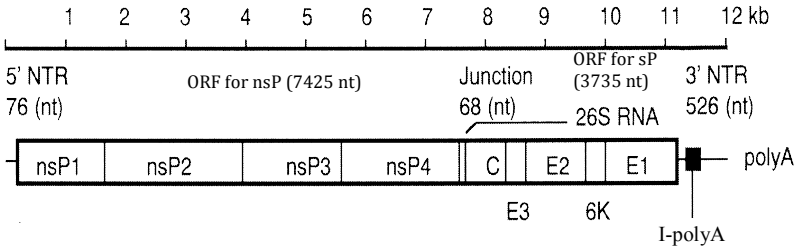

Figure 4.1 Schematic presentation of the CHIKV genomic RNA, genes and translated proteins. The scale indicated is in kilobases (kb), and presented here are the following 5 regions: the 5'NTR of 76 nt, the ORF of 7,425 nt that translate non-structural proteins, the junction region of 68 nt, and the ORF of 3,735 nt (or 26S subgenomic RNA) that translate structure proteins and the 3'NTR of 526 nt. Reproduced and adapted from Journal of General Virology [100].

As for other Alphaviruses, the replication cycle of CHIKV (Figure 4.2) starts by virus infiltrating the host cell by endocytosis. The E1 peptide, which mediates virus-host cell membrane fusion, is exposed following conformational changes triggered by low pH of the endosome's environment. Thereafter, the viral genome, from which two precursors of nsP translated from the viral mRNA undergo cleavage to generate the four nsP (nsP1, nsP2, nsP3 and nsP4), is released. These nsP play an important role in the replication process of the virus. The nsP1 is involved in synthesis of negative-stranded viral RNA intermediate; nsP2 displays RNA helicase function, as well as RNA triphosphatase and proteinase activities. This protein also plays a role in the viral shut-off of host cell transcription. The nsP3 is a part of the replicase unit whereas nsP4 functions as the viral RNA polymerase. Together, these proteins form the viral replication complex that synthesizes the negative-stranded RNA intermediate which acts as template for the transcription of the subgenomic (26S) and genomic RNAs. The subgenomic RNA is then translated to the precursor of sP, the C-pE2-6K-E1, which is processed by an autoproteolytic serine protease thus releasing the capsid protein (C). The pE2 and E1 proteins associate in the Golgi and move to the plasma membrane where pE2 is cleaved into E2 involved in receptor binding, and E3, which mediates proper folding of pE2 and its association with E1. The recruitment of the membrane-associated envelope glycoproteins to the encapsidated viral RNA form the virion, with an icosahedral core, which buds at the cell membrane [101].

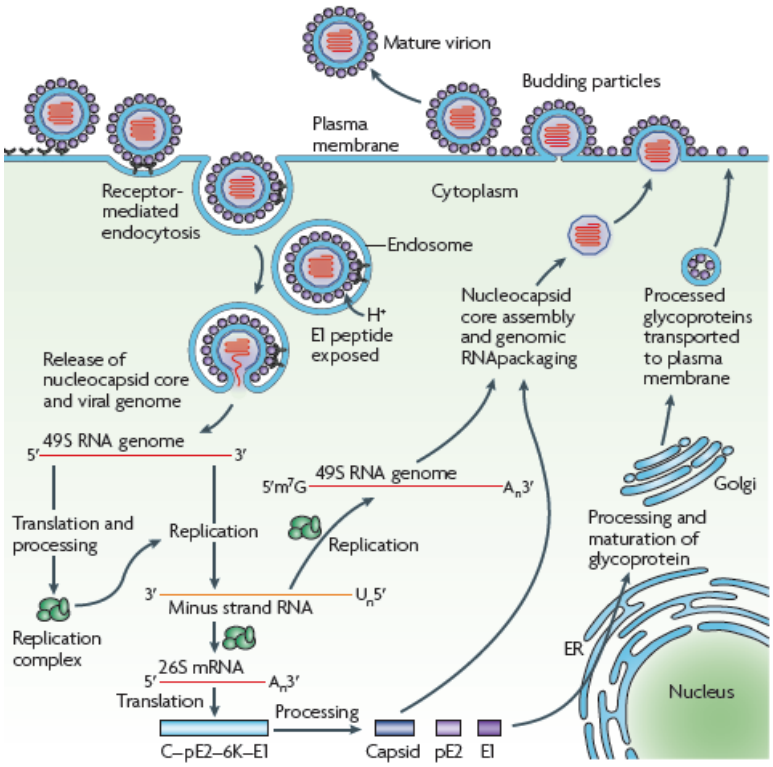

Figure 4.2 Replication cycle of alphaviruses [101]. Reproduced with permission requested from Springer Nature.

1.4.2 Transmission, clinical presentation and epidemiology

CHIKV is almost exclusively transmitted by mosquitoes. This vector maintains the sylvatic transmission cycle between non-human primates and mosquitoes, especially in Africa. A human-mosquito-human transmission cycle has been reported from Asia, the Indian Ocean, Africa, and from Europe. In addition to humans, other known hosts of CHIKV are monkeys, rodents and birds. *Aedes aegypti* mosquitoes were reported to be responsible for outbreaks within the tropical regions, while *Aedes albopictus* transmitted the virus in temperate areas. Mother-to-child or vertical transmission and blood transfusion were also reported as potential mode of transmission of CHIKV [102-104].

CHIKV is associated with a high rate of infection of a given population. Within a community affected by an outbreak, 10%-70% of individuals become

infected, with 50%-97% of these presenting clinical manifestations. In addition, high seroprevalence rates were recorded from areas that experienced different outbreaks: 38.2% positive subjects for specific IgM/IgG were found in the Indian Ocean island of La Reunion, 75% in the island of Lamu in Kenya, 63% in Grande Comoro island and 90.4% in Suriname [105-109]. Clinical signs associated with CHIKV infection are polyarthalgia, which may persist for years, fever, transient macular or maculopapular rash found mainly on extremities, trunk and face, headache, and back pain [102]. CHIKV fever is also associated with severe complications such as neurological (encephalitis, Guillain-Barré Syndrome, etc), and haemorrhagical conditions such as clotting abnormalities or severe thrombocytopenia. Other reported complications are conjunctivitis, myocarditis, pneumonia, nephritis, hepatitis, pancreatitis, etc. Severe infection may lead to death, often due to multiple organ failure [104].

CHIKV is endemic in tropical Africa, South-East Asia and in the Indian subcontinent.

Since its first recorded outbreak in the Newala District, Tanzania, in 1952, CHIKV outbreaks have expanded (Figure 4.3 A & B) to Senegal in 1996-1997, Malaysia in 1998-1999, the Central Africa Republic and the DRC in 1999-2000, to Indonesia in 2000-2003, and the Indian Ocean islands and India in 2004-2007. This latter outbreak, which occurred in a novel climate zone, was reported to be more severe and associated with a new vector species, *Aedes albopictus,* while previous outbreaks were vectored by *Aedes aegypti.* Three genotype groups of CHIKV, based on the sequencing of the envelope protein E1, have been described. These are the east-central-south-African (ECSA) genotype, the Asian genotype and the west-African genotype [110, 111].

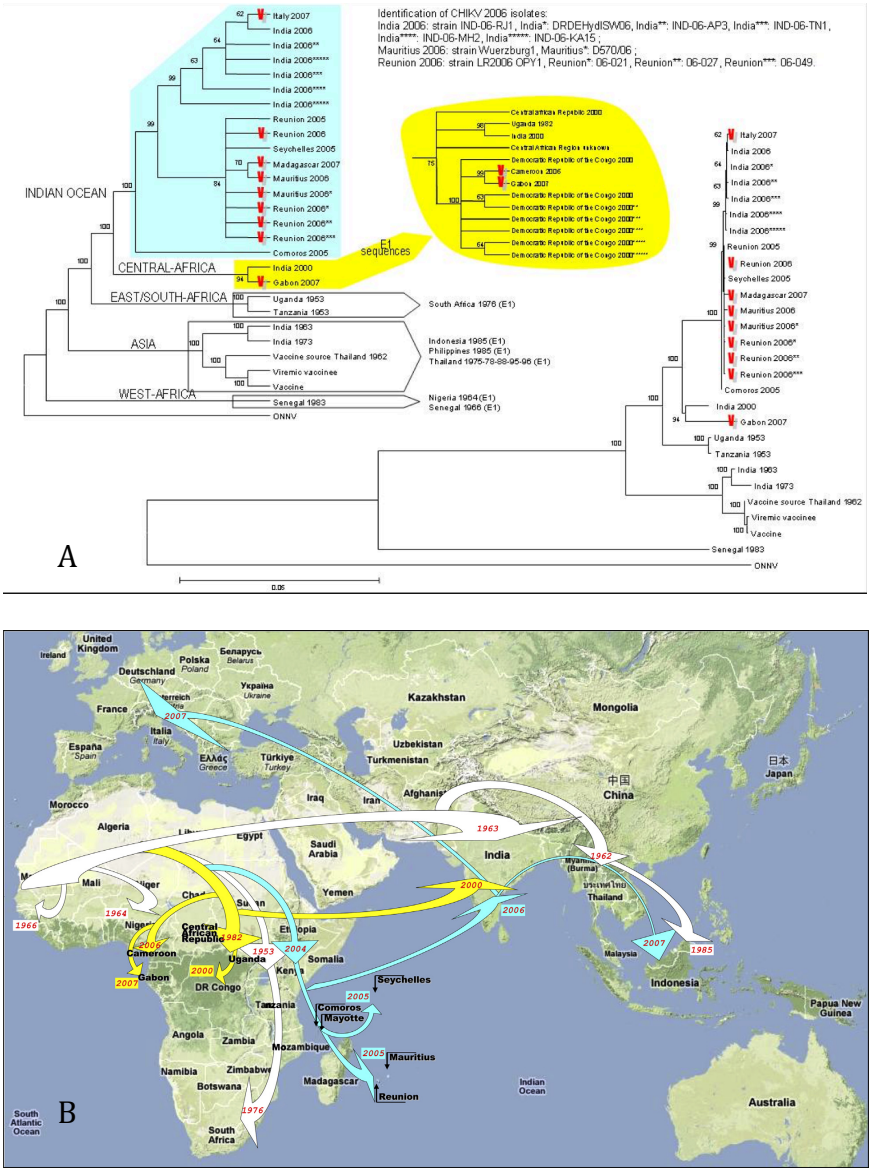

Figure 4.3 Suggested dispersal of CHIKV from Africa to the Indian Ocean and Europe during the past 20-50 years. Viral evolution and spread are represented according to recent phylogenetic studies. Different evolutionary lineages are identified using specific colours on the phylogenetic tree (A) and arrows with specific colours on the global map (B) [112].

1.4.3 Diagnosis

Laboratory analysis of serum samples from suspected patients, by serology and/or molecular methods, confirms the diagnosis of CHIKV infection. The RT-PCR and virus culture might frequently be positive in samples that are collected between 3 and 10 days of the acute phase of the disease. Anti-CHIKV IgM antibodies are detectable during the acute phase that lasts for 14 days from the onset of the disease, but might also be positive in samples collected up to 3 months from onset, while IgG antibodies may persist for years [113].

Other laboratory abnormalities observed may include lymphopenia, thrombocytopenia, leucopenia, anemia, increased liver enzymes, creatinine and creatinine kinase, and hypocalcemia [104].

In May 2015, experts concluded on CHIKV case definition, based on different phases of the disease. Four phases of CHIKV infection were described [114]:

1. The acute clinical case is characterized by fever > 38.5°C and joint pain (usually incapacitating). These symptoms are often accompanied by exanthema, myalgia, back pain, headache, or vomiting and diarrhea. The latter symptoms are common in children with acute onset of the disease. Furthermore, epidemiological criteria such as being a visitor or a resident in an area where local transmission of CHIKV has occurred during the preceding 15 days, or laboratory criterion of confirmation by PCR, serology or viral culture, should be fulfilled.
2. Atypical clinical cases of CHIKV infection, confirmed by laboratory diagnostics, include other manifestations such as neurological, cardiovascular, dermatological, ophtalmological, hepatic, renal, respiratory, or hematological symptoms.
3. Severe acute cases are defined as infected patients with laboratory-confirmed CHIKV with dysfunction of at least one organ or system that threatens life and requires hospitalization.
4. Suspected and confirmed chronic cases are defined as having a clinical diagnosis of CHIKV after 12 weeks following the onset of symptoms (presented continuously or recurrently) such as pain, rigidity, or edema. Its confirmation is laboratory based.

1.4.4 Treatment and prevention

As there is no antiviral treatment against CHIKV only symptoms can be treated. Disease-modifying anti-rheumatic drugs such as methotrexate, hydroxychloroquine or sulphasalazine might ameliorate severe chronic arthritis;

but treatment with anti-TNF-α antibodies was also associated with successful results. Several attempts in producing a vaccine and specific treatment agents are ongoing [115], but have hitherto not been successful.

Prevention consists of implementation of vector control measures such as the use of mosquito repellents and cleaning of mosquito breeding sites [104, 109].

1.5 West Nile virus

1.5.1 Virology: genes, proteins and replication

West Nile virus (WNV) is a neurotropic mosquito-borne virus, which was reported for the first time in 1937 from a febrile patient from the West Nile district in Uganda [116].

WNV is a single-stranded, positive-sense RNA virus, which by taxonomy is placed in the *Flavivirus* genus within the *Flaviviridae* family. Other genera in this family are *Pestivirus* and *Hepacivirus*, the latter to which the important pathogen hepatitis C belongs. WNV is a member of the Japanese encephalitis virus serocomplex of flaviviruses which include Japanese encephalitis, Kunjin, Murray Valley encephalitis, St. Louis encephalitis, Kokobera, Alfuy, Stratford, Usutu and Koutango viruses. Other, more distantly related flaviviruses that are important human pathogens, are Dengue, yellow fever, and tick-borne encephalitis viruses (TBEV) [117].

The viral genome, of around 11 kb with untranslated regions (UTR) at both ends, contains 10 genes (Figure 5.1) that encode one polyprotein subjected to cleavage into 3 structural proteins [the capsid (C), premembrane/membrane (prM/M) and envelope (E) proteins], and 7 nonstructural proteins (NS) known as the NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 [118].

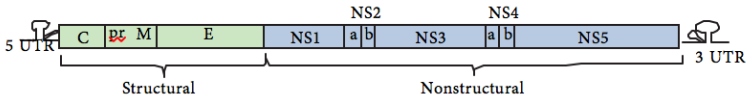

Figure 5.1 WNV genome representation with one ORF encoding 3 structural and 7 nonstructural proteins [118].

During the replication process, the viral envelope (E) protein binds to the host cell receptors, and virions (Figure 5.2) enter the cytoplasm by receptor-mediated endocytosis. Virion particles are then transferred to the endosomes where they mature through a conformational change of the viral E protein dimer effected by the low pH within the endosomes. Fusion of viral particles with endosomal membrane triggers the release of WNV genomic RNA that initiates translation of polypeptides, and transcription into a complementary, negative-sense RNA intermediate, from which many new viral RNA molecules are replicated. These genomic viral RNA molecules are encapsidated by coupling with the C protein before budding into the endoplasmic reticulum while recruiting prM and E proteins. These events occur within secluded replication complexes, which are formed from ER membranes, and involve upregulation of enzymes involved in cholesterol metabolism [119]. Immature virions are brought through the host secretory pathway by intracellular vesicles that fuse with the plasma membrane and release mature virions by exocytosis [120, 121].

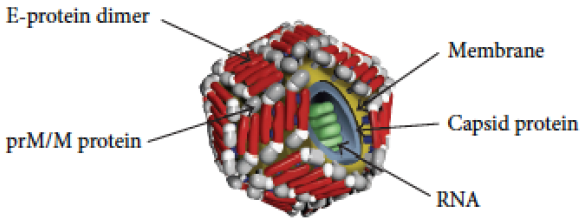

Figure 5.2 Structure of WNV virion [118].

1.5.2 Transmission, epidemiology and clinical presentation

Culex mosquitoes transmit WNV while feeding on infected birds, which are considered as amplifying hosts. WNV is then passed on to susceptible vertebrates including humans and horses as accidental dead-end hosts (Figure 5.3). Blood transfusion and organ transplant are also reported as a route for transmission, which in addition may occur from mother to child *in utero* or through breastfeeding [122]. Migratory birds were reported as transport vehicles of the virus from sub-Saharan Africa to Europe, America and beyond [123]. Some reports also suggest that some ticks, namely Argasidae *Argas* and Ixodidae *Hyalomma* species, participate in transmission of WNV [124-126].

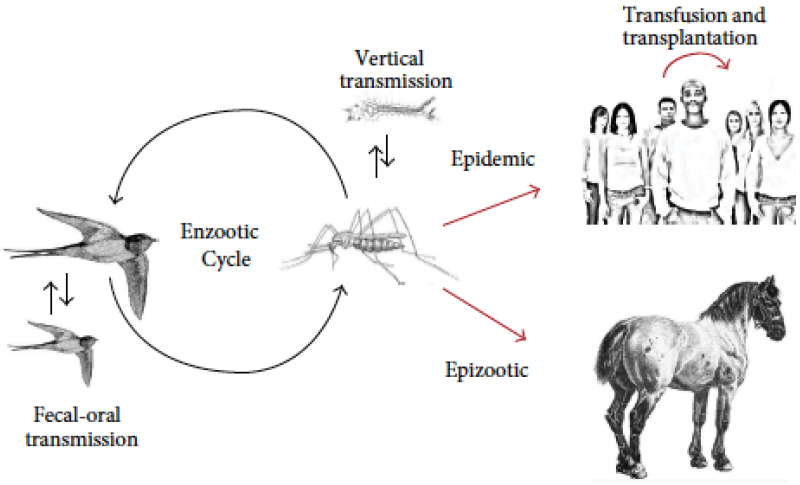

Figure 5.3 WNV transmission cycle. Enzootic amplification of WNV occurs by birds and mosquitoes, by bird-to-bird transmission and through transmission between cofeeding mosquitoes. Vertical transmission by mosquitoes provides a mechanism of virus overwintering. Humans and horses are regarded as accidental dead-end hosts. Human-to-human transmission may occur through blood transfusion and organ transplant [118].

WNV is distributed worldwide (Figure 5.4) with multiple viral lineages defined according to nucleotide differences detected through genomic sequencing. Lineages 1 and 2 are the most commonly identified in Africa, where lineage 1 is frequent in central and northern Africa, Europe, Australia and Americas, and lineage 2 endemic in southern Africa, Madagascar, central Europe. Lineages 3 and 4 are found in Russia, lineage 5 in India, and the less common lineages 6, 7 and 8 respectively in Malaysia, Senegal and Spain. Virulence severity depends on the genotype. Lineages 1 and 2 were associated with epidemics within North America and Europe [124, 127-129].

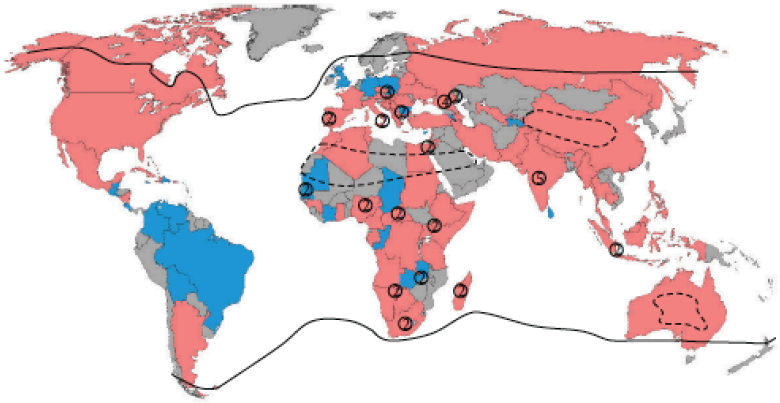

Figure 5.4 Global distribution of WNV by country: Red-human cases or human seropositivity; Blue-non-human/mosquito cases or seropositivity; Gray-no data or no positive reported. Black lines represent worldwide distribution of the main WNV mosquito vectors, excluding areas of extreme climate denoted by dashed lines. Circled numbers indicate the reported presence of WNV lineages other than lineage 1 in that specific area [118].

In most of the human cases, WNV infection is asymptomatic. Only 20% of infected individuals manifest clinical signs such as fever, headache, rash, muscle pain or weakness, joint pain or hepatitis. Neurological complications such as encephalitis, meningitis, Guillain-Barré syndrome occur in less than 1% and are associated with 10-20% of mortality, while survivors may develop severe neurological anomalies [130, 131]. Beside humans, horses are the most commonly infected mammal, where 90% of the affected animals develop neurological manifestations with a mortality rate of 30% [132].

1.5.3 Diagnosis

WNV infection is confirmed serologically by detection of IgM antibodies in serum and/or CSF using ELISA. IgM antibodies are often detected at the onset of neurological signs. Increased IgG antibodies against WNV measured from sera during the acute and convalescent phase are also indicative of WNV infection (Figure 5.5).

IgM antibodies may, in some cases, be detected persistently until a year after onset; this is why IgM findings are not always indicative of an acute infection.

Moreover, the WNV ELISA test does not discriminate IgM antibodies against WNV from those of St. Louis encephalitis or Japanese encephalitis vi-

ruses. For confirmation, we refer to the neutralization test in form of the PRNT [124].

It is difficult to isolate WNV from the blood, or to detect viral RNA by RT-PCR, as the viremia in humans and in horses is transient and of low quantity. However, in cases with encephalitis, findings of WNV RNA from the CSF have been reported, so RT-PCR performed on this body fluid may contribute to the diagnosis [133].

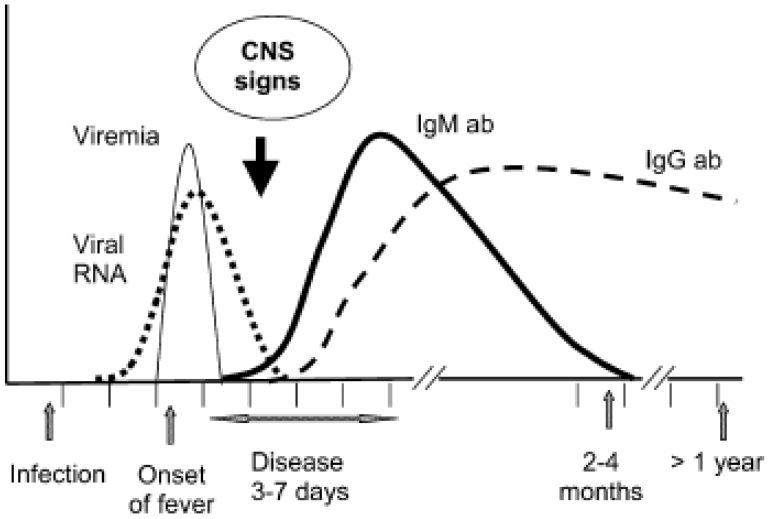

Figure 5.5 Viremia and antibody kinetics in West Nile virus infection [124].

1.5.4 Treatment and prevention

At present there are no available treatments or vaccines against WNV. Vector control is currently the main means for prevention of WNV infection. Control is carried out by avoiding exposure to infected mosquitoes, by cleaning breeding sites, and by the use of larvicides, spraying pesticides, and insect repellents. In endemic areas, screening of blood donation could be recommended using RT-PCR [122].

1.6 Zika virus

1.6.1 History

During a thorough search for viruses related to yellow fever virus, Zika virus (ZIKV) was originaly isolated in 1947 from the serum of a rhesus monkey from the Zika forest in Uganda. One year later an identical virus was isolated from homogenates of *Aedes africanus* mosquitoes from the same forest[134]. Thereafter, several regions reported their seroepidemiological data on ZIKV including, in Africa: the Central African Republic, Egypt, Gabon, Kenya, Nigeria, Senegal, Sierra Leone, Tanzania and Uganda, and in Asia: India, Indonesia, Malaysia, Pakistan, the Philippines, Thailand, and Vietnam. Before the outbreak of ZIKV on Yap island in the Federate States of Micronesia in 2007, ZIKV was seldom considered as a major pathogen as it was previously associated only with mild self-limiting febrile disease within those endemic areas [135]. However, during the 2007 outbreak, a majority of the population fell ill with high fever, rash, artritis, conjunctivitis, headache, edema and vomiting [136].

1.6.2 Virology: genes, proteins and replication

ZIKV is a zoonotic mosquito-borne virus belonging to *Flavivirus* genus within the *Flaviviridae* family. This virus was recently associated with major severe outbreaks and rapid global spread to non-endemic areas. The clinical presentation, which attracted high medical and media attention, was characterized by neurological complications and congenital malformations [137].

Genetically, ZIKV carries a single-stranded, positive-sense, nonsegmented linear RNA genome of about 10,807 nucleotides (Figure 6.1.A). This genome contains a single ORF, which is flanked by two non-coding regions (NCR), and encodes a polyprotein of 3,423 amino acids. After translation, this polyprotein is cleaved into three structural proteins: C, prM, and E, and seven nonstructural proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5. Cleavage is brought about by the action of a combination of four proteases (Figure 6.1.B): the host signal peptidase, the viral NS3 serine protease, the host furin-like protease and an unknown host protease [135].

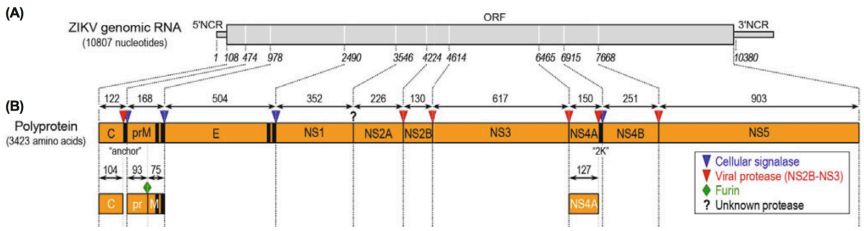

Figure 6.1 Genome structure and polyprotein processing of ZIKV [135]. (A) Genome structure. The positive-sense genomic RNA of ZIKV is composed of a 5'NCR, a single long ORF, and a 3'NCR. (B) Polyprotein processing. The viral ORF encodes a 3,423 amino-acid polyprotein, which is co- or post-translationally processed by host- and virus-encoded proteases into structural and non-structural proteins. Vertical black bars represent one or two transmembrane domains. Also indicated are the putative cleavage sites conserved among flaviviruses and the lengths of the cleavage products. Reproduced with permission from Springer Nature.

The replication of ZIKV, as for many other flaviviruses, starts by attachment of virions to the host cell surface using adhesion factors such as lectin DC-SIGN and members of the phosphotidylserine receptor family. The viral E glycoprotein interacts with cellular receptors and triggers entry of the virus into the cell, by rapid clathrin-mediated endocytosis. In the endosomes, viral and cellular membranes merge due to the acidic environment in this compartment. After fusion of membranes, virion releases the genomic RNA, which enters the cytosol and serves as mRNA, translating the polyprotein in association with the ER. Thereafter, the positive-sense RNA genome is transcribed into its complementary negative-sense RNA that in turn serves as a template for synthesis of many new positive-sense viral RNA genomes with involvement of different nonstructural proteins that also play a role in viral assembly. Viral glycoproteins prM and E form a heterodimer on ER membrane, for viral assembly, and drive the budding of the viral genomic RNA and C proteins into the ER lumen forming the immature virions. These particles move through the cellular secretory pathway during which the *trans*-Golgi-resident furin or furin-like protease cleaves prM into M protein and "pr" peptide leading to major structural rearrangements of the M and E proteins with formation of mature virions that are transported to the cell surface and released by exocytosis [135].

1.6.3 Transmission, clinical presentation and epidemiology

ZIKV is transmitted to vertebrate hosts including humans by the bite of *Aedes* mosquito species, in particular *Aedes albopictus* and *Aedes aegypti* [138]. In contrast to WNV, humans can amplify ZIKV so that might maintain a human-to-human transmission cycle as well (Figure 6.2). Non-vector transmission modes of ZIKV include blood transfusion, organ transplantation, sexual intercourse, and vertical transmission (from mother to child) [139, 140].

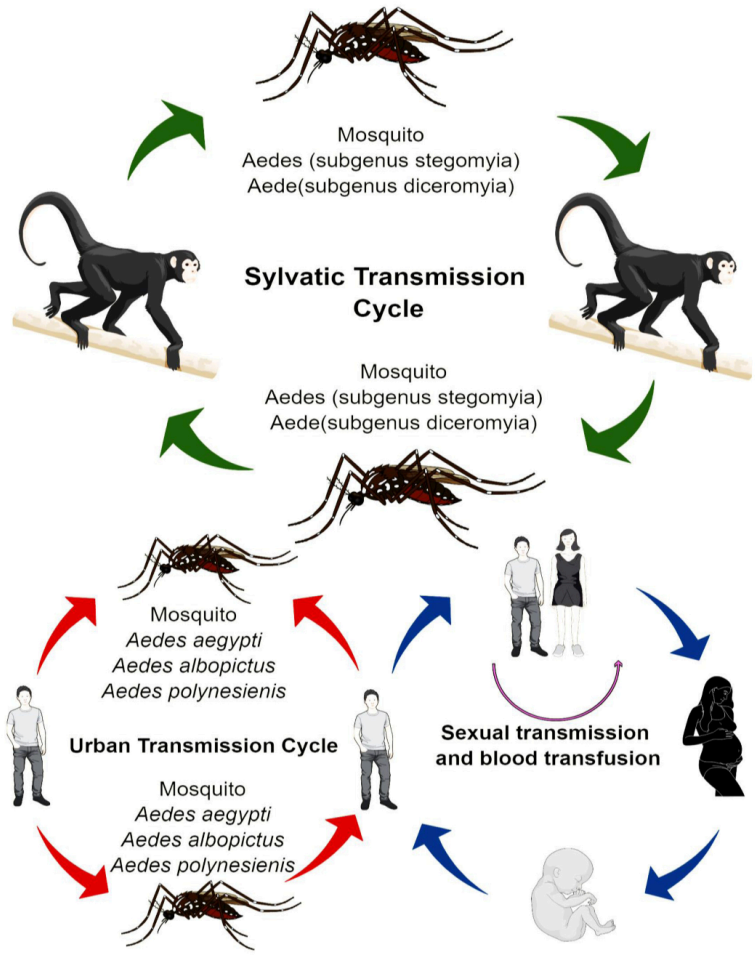

Figure 6.2 Transmission cycle of ZIKV [140].

Clinically, ZIKV infection is manifested by mild fever, headache, myalgia, arthralgia, conjunctivitis, prostatitis, hematospermia, subcutaneous bleeding and a rash, that often resolve within 3-7 days [141]. The disease may cause severe complications such as congenital malformations, in particular microcephaly (Figure 6.3), or neurologically by the autoinflammatory reaction Guillain-Barré syndrome [140].

Figure 6.3 ZIKV and microcephaly. Source: CDC.

From 1947 to 2007, though ZIKV infection was progressively expanding geographically (Figure 6.4), it was still considered as a benign disease with only 16 human cases sporadically recorded and no related death reported. This figure might be underestimated since similar clinical manifestations are presented by dengue and/or chikungunya viruses' infections, two viruses that are prevalent within the tropical region of Africa and Asia [142].

The new era of severe infection due to ZIKV was first recognized during one of the largest human outbreaks to be recognized; this was on the Pacific island of Yap in the Federated States of Micronesia where 73% of inhabitants older than 3 years were found to be infected [136]. In 2015, Brazil reported 138 cases with neurological manifestations, of these 42% had a history of viral infection with 55% showing symptoms consistent with Zika or dengue infection [137].

Three genotypes of ZIKV were identified (Figure 6.5) with older strains belonging to the East African genotype, followed by the West African line, and, recently, the Asian genotype which has spread eastward through the Pacific region to the American continent. Most likely, some genetic alterations within the Asian genotype can explain the recent emergence and global spread of ZIKV.

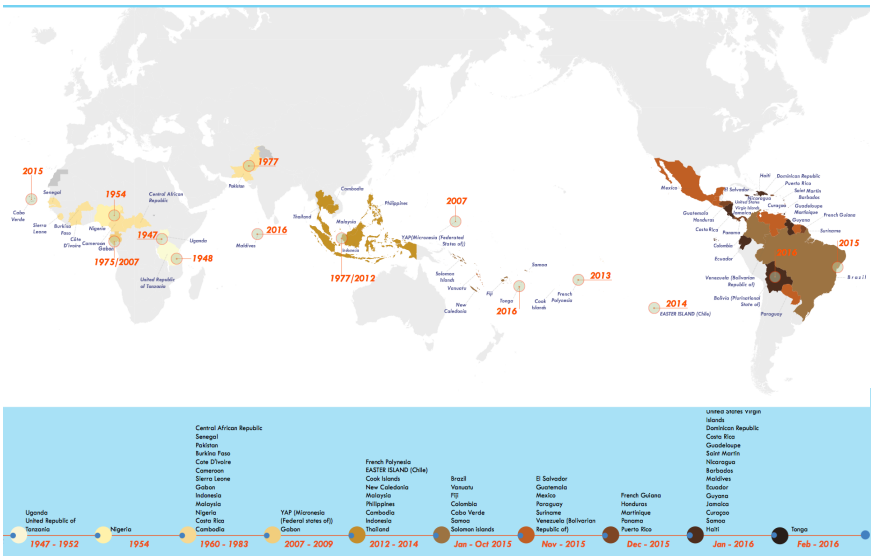

Figure 6.4 Countries and territories showing historical time-line of Zika virus spread (1947 - 2016). Source: WHO.

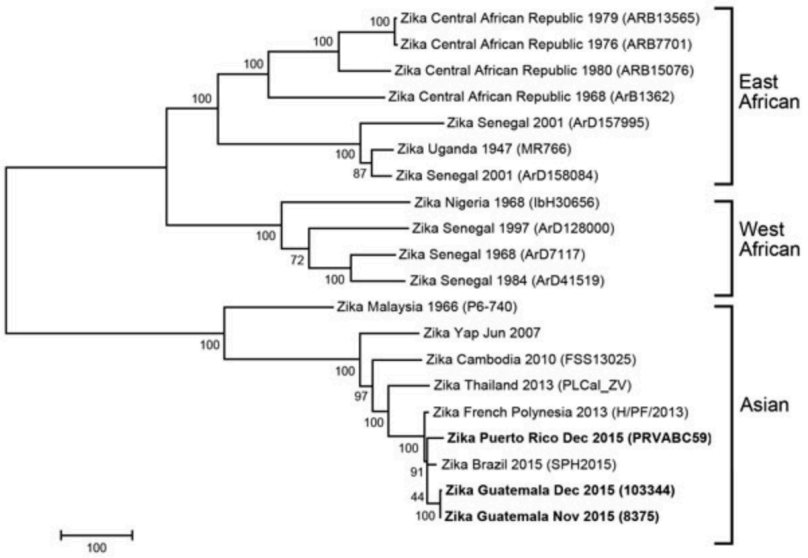

Figure 6.5 Phylogenetic tree of Zika virus strains identified from Guatemala and Puerto Rico in December 2015 (indicated in boldface) compared with reference isolates obtained from GenBank. Scale bar indicates number of nucleotide substitutions per site. Source: CDC.

1.6.4 Diagnosis

ZIKV can be readily diagnosed by use of the ELISA that detects anti-ZIKV IgM antibodies in serum or other body fluids, including saliva. From reports of other flaviviruses, IgM may be detected within 10 days, and up to 2 months after the onset of clinical signs. However, ELISA tests are not always reliable due to cross-reactivity with other flaviviruses, and the plaque reduction neutralization test (PRNT) is sometimes used to confirm the specificity of ZIKV-IgM signals [143].

Due to the problems of serological specificity, the diagnosis of ZIKV often relies on RT-PCR methods, which can detect the viral RNA within 1 week of the onset of clinical manifestations [143]. Suitable sample fluids are serum and plasma, whole blood, urine and amniotic fluid.

1.6.5 Treatment and prevention

At present, ZIKV treatment is based on management of symptoms only, as no ZIKV vaccine or licensed antivirals are available for prevention or treatment. In parallel with the situation for several other flaviviruses, research is ongoing towards development of vaccines and antivirals drugs against ZIKV [144-146].

Prevention of ZIKV infection consists mostly of mosquito vector control by eliminating breeding sites by distribution of larvicides, by the use of mosquito repellents and bed nets. Human-to-human spread can be reduced by avoiding unprotected sex with an infected partner, but also by preventing exposure of the pregnant women to ZIKV [147].

1.7 Tick-borne encephalitis virus

1.7.1 Virology: genes, proteins and replication

Tick-borne encephalitis, as a clinical entity, was reported for the first time in Europe in 1931 from an outbreak of "acute epidemic serous meningitis" in southeast Austria, and the virus was first isolated in the former Soviet Union in Russia in 1937 [148, 149].

Although TBEV belongs to the flaviviruses, as a tick-borne virus it forms a separate genogroup together with viruses such as Omsk hemorrhagic fever virus (OHFV), Powassan virus, Alkhurma virus, and louping ill virus. In general, the group of tick-borne flaviviruses has been given less scientific attention as compared to the mosquito-borne flaviviruses [150].

The TBEV genome, as that of ZIKV, consists of a linear positive-sense, single-stranded RNA with only one ORF encoding one polyprotein that is cleaved into three structural proteins, the envelope (E), the capsid (C), the membrane (M), and seven nonstructural proteins [150].

Replication of the virus starts by virus attachment and penetration into the host cell through receptor-mediated endocytosis where the viral E protein attaches onto host cell receptors such as heparan sulphate and other glycosaminoglycans, which are commonly found on many cell types of vertebrates and ticks [151]. The fusion of the virus-containing vesicle with the endosomes, within an acidic environment, triggers a conformational change of viral envelope E protein inducing the release of the viral nucleocapsid into the cytoplasm of the host cell. Thereafter, the viral RNA is translated directly by host cell ribosomes to viral polyproteins that are cleaved by viral serine protease and cellular proteases into the respective viral proteins. The transcribed negative-sense viral RNA serves as a template for transcription of new copies of the positive-sense viral RNA genome. These positive-sense RNA molecules are encoated by viral capsid proteins to form nucleocapsids that bud into the ER lumen. Here, the nucleocapsids are covered by a bilayer lipid membrane with prM and E protein molecules inserted, forming immature virions. These virions are transported to the Golgi vesicles through the secretory pathway where they mature with cleavage of the prM protein by the host cell protease furin. Fusion of mature virions with plasma membrane of the host cell triggers the release of the virus through exocytosis [152, 153].

1.7.2 Transmission, clinical presentation and epidemiology

TBEV is usually transmitted by ticks of the Ixodideae family. Furthermore, unpasteurized and virus-contaminated diary products such as raw milk and cheese from goats and sheep could infect susceptible individuals through the alimentary tract [154-156]. The life cycle of TBEV is normaly maintained by hard ticks (*Ixodes ricinus* and *Ixodes persulcatus* mainly) functioning as vectors, and by small rodents (Myodes Glareolus), hares and deer acting as reservoirs (Figure 7.1). Humans and domestic animals (cattle, goat, and sheep) are considered as dead-end hosts that are unable to transmit the virus due to their inability to develop a high grade of viremia [157]. Migratory birds have been suggested as transporters of the virus over large distances, most likely by carrying TBEV-infected ticks into new areas, where they can expand far from their original endemic foci [158].

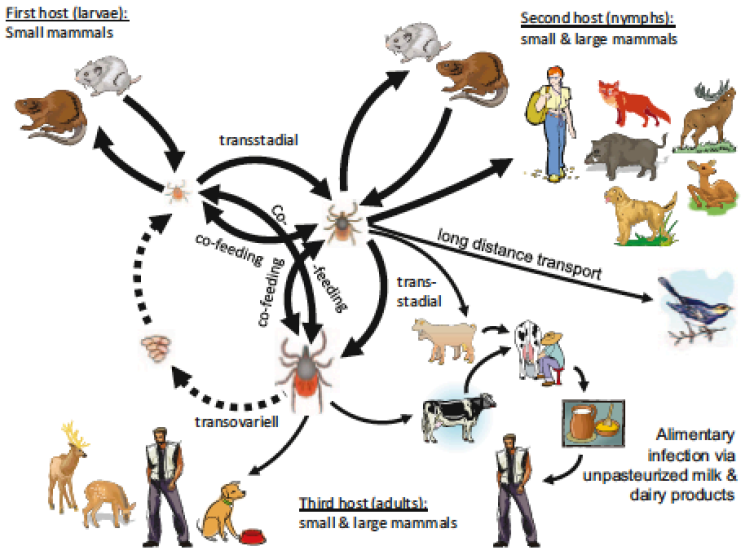

Figure 7.1 Developmental stages of Ixodes ricinus (Ixodes spp.) with TBEV transmission routes [159]. Reproduced with permission requested from Springer Nature.

Most often TBEV infection is asymptomatic. However, when the infection does present with clinical signs and symptoms, these are typically of a neurological character such as encephalitis, meningitis or meningo-encephalitis with or without paralysis, and other neurological sequelae [160]. Common clinical symptoms are chills, headache, nausea, vomiting, muscle pain, sore throat,

blurred vision, photophobia, poor concentration, tremor, etc [154, 156]. The viral subtype may influence the severity of TBE. For the European subtype (TBEV-Eu), up to 10% of patients can develop neurological sequelae, with a mortality rate of 0.5-2%, as compared to the more virulent Siberian subtype (TBEV-Sib) for which the infection may have a longer duration and where 2-3% of patients die. The Far-Eastern subtype (TBEV-Fe) is reported to be even more severe, with a case fatality rate of up to 40% and high rate of neurological sequelae in survivors [161]. However, it should be mentioned here that more recent studies from China and Japan report much lower mortalities of this subtype [162].

These three subtypes manifest a low variation of their respective amino acid sequence, with 2.2% variability within subtypes, and up to 5.6% between subtypes when sequencing the gene that encodes the viral envelope E glycoprotein. This protein induces a high amount of neutralizing antibodies, as compared to other viral proteins such as NS1. The protective IgG response of the host is active against all TBEV subtypes (genotypes), thereby classifying TBEV as monoserotype virus [152, 163].

TBEV is reported to be endemic in central, eastern and northern Europe and Asia (Figure 7.2.A). Most TBEV cases occur during the period of activity of ticks [164], often from April to November. The three main genetic subtypes (as mentioned above), which usually are based on envelope protein E sequencing, are each more or less prevalent in different endemic foci. However, multiple subtypes may sometimes be detected within an individual endemic area. The TBEV-Fe subtype is predominantly found in far-eastern Asia and Japan, while the TBEV-Eu subtype is highly prevalent in Europe. In addition, a third genotype (TBEV-Sib) is endemic in Siberia, the Baltics, and northern Finland (Figure 7.2.B). Two additional genotypes were also identified in eastern Siberia, but too few sequences have been presented to allow a characterization of these viruses as possible new subtypes. The future taxonomy may well be based on numerical genotypes instead of geographical names, since a more mosaic pattern of spread can be anticipated. For example, a recent report describes the finding of TBE-Eu in several locations on the Korean peninsula [162]. An example of such a numerical classifications is given in Figure 7.2.B.

Cases decreased drastically in Europe where 12,733 were recorded in 1996 and only 2,876 in 2016 within all the European regions [161, 165-167].

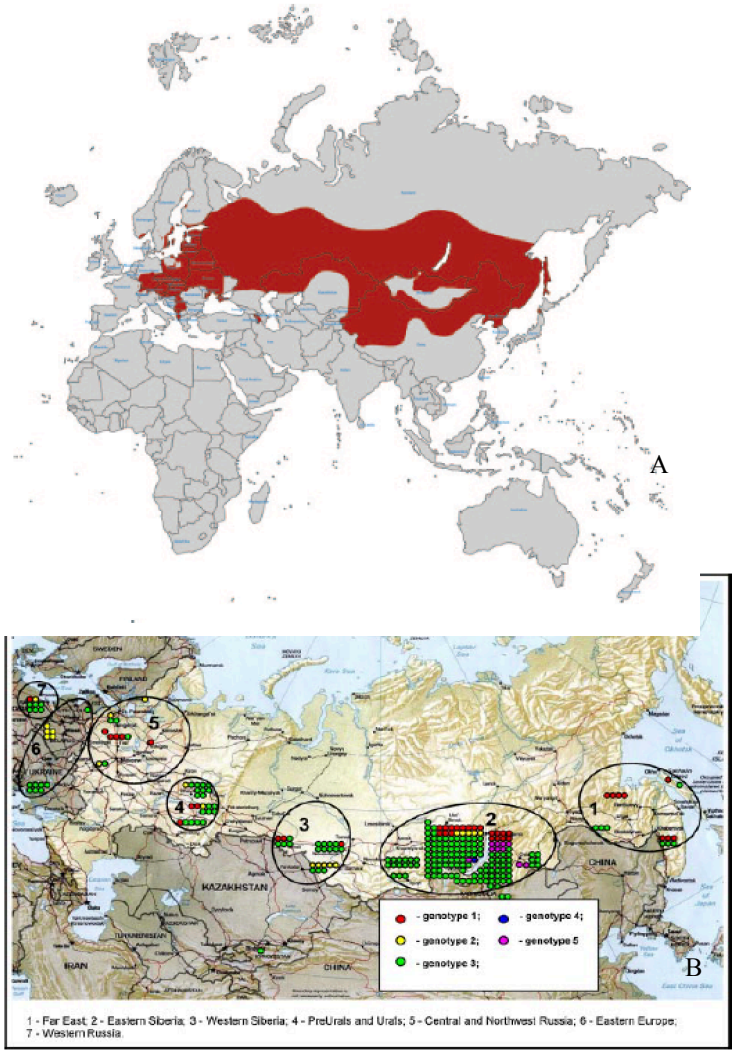

Figure 7.2 ***A.*** *The "TBE belt" (prevalence of TBE viruses) in ticks, animals, and humans [159].* ***B.*** *Geographical distribution of TBEV genotypes [167]. Reproduced with permission from the Journal of Medical Virology.*

1.7.3 Diagnosis

Diagnosis of TBE infection is demonstrated serologically through ELISA by detection of IgM and IgG antibodies from serum and CSF samples [156, 168]. IgM antibodies may be detected for months whereas IgG antibodies can prevail for a long time and may confer lifelong immunity [169].

To overcome diagnostic bias due to extensive cross-reactivity among flaviviruses, the ELISA tests can be confirmed by a neutralization test [170, 171].

Viral RNA can also be detected by RT-PCR in blood, urine or CSF collected mainly during the first viremic period before seroconversion and occurrence of CNS inflammation. If PCR sampling is delayed, the method has less diagnostic value [156, 168, 169, 172].

1.7.4 Treatment and prevention

There is no antiviral treatment against TBEV. Thus, patient care is based on symptomatic therapeutics.

Post-exposure prophylaxis by immunoglobulins containing gamma globulin against TBEV scheduled within 96 hrs of tick bite was discouraged due to unreliable protection and to reports about induction of a more severe course of the disease in children [173].

The prevention instead focuses on promoting the awareness of the risk of infection from tick bites in TBEV-infested regions, and vaccination of subjects living in or travelling to endemic areas is encouraged. Two killed vaccines, both of which are based on the TBEV-Eu subtype of viruses, are commercially available for children and adults in Europe: FSME-Immun® approved by the European Medicine Agency since 1976 and Encepur® from 1991. Both vaccines were found to confer 100% seroconversion with minimal adverse reactions. After introduction of general vaccination in Austria, a sharp decline in the TBE incidence was reported [174]. The vaccine is scheduled in 3 doses, from Day 0, after 1-3 months, and the third after 9-12 months, with boosters recommended every 3-5 years. Similarly, two Russian vaccines, based on the TBEV-Fe subtype, TBE-Moscow and EnceVir, are mostly used in Russia and China [149, 155]. However, a cautionary warning on the efficacy of killed TBEV vaccines should be raised, based on recent reports of a substantial number of TBE cases with vaccine break-through infection [175]

2. Aims

2.1 General aim

To utilize seroepidemiology in Rwanda to control for immunity to emerging arboviral infections, and to exanthematous viral infections that can be prevented by vaccination programs.

2.2 Specific aims

Paper I

To assess the seroprevalence of MeV in Rwanda, and to relate these findings to the reported vaccine coverage and to the incidence of clinical measles in the country.

Paper II

To investigate, through seroepidemiology, the susceptibility of Rwandans to two viruses that cause congenital lesions: RuV, a vaccine-preventable infection, and ZIKV, an emerging flavivirus.

Paper III

To assess the seroprevalence of CHIKV and WNV in Rwandan blood donors, and the prevalence of the mosquito vectors used by respective viruses.

Paper IV

To analyse linear epitopes of sera that were cross-reactive between WNV and TBEV among Rwandan and Swedish blood donors.

3. Materials and Methods

3.1 Study design and samples collection

The study was designed as a sandwich PhD project where half of the time was dedicated to fieldwork in Rwanda and the other half to laboratory analyses and PhD courses in Sweden (Figure 8.1)

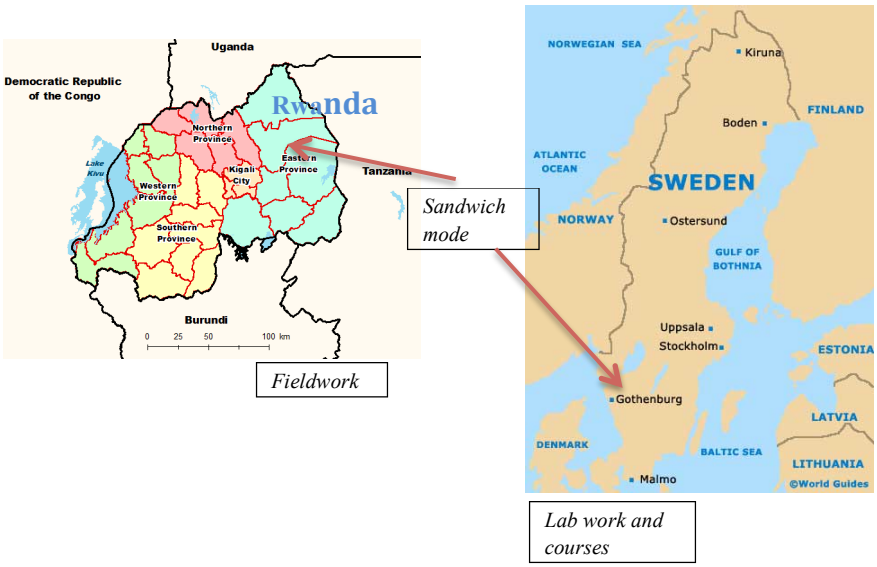

Figure 8.1 Study project design representing the map of Rwanda and of Sweden showing Gothenburg

The fieldwork consisted of collection of plasma samples from blood donors from all four provinces of Rwanda, and from the City of Kigali.
The National Center for Blood Transfusion (NCBT) in Rwanda comprises 5 regional centers: Kigali, Rwamagana, Huye, Musanze and Karongi. Each of the-

se regional centers has a blood collection center, in addition to the 548 mobile collection sites that are active nationwide [176].
Recruitment of blood donors was conducted from March to October 2015, in line with the policy of the Rwanda National Center for Blood transfusion [177]. The following data were recorded from the donors: age, sex, and residence. Blood samples of 3-5 ml whole blood in EDTA tubes were collected from each participant. Samples were then transported to the corresponding regional center for blood transfusion where they were centrifuged for separation of 0.5 to 1.5 ml of plasma, which was stored directly at -20^{o}C. All plasma samples from each region were then transferred to the Rwanda Military Hospital, located in Kigali City, and kept frozen at -80^{o}C until shipment to Sweden.

Serum samples from Swedish blood donors were anonymous, and characterized by age and sex only. These samples were also collected in September 2015, and stored frozen at -20^{o}C in the clinical laboratory of the Microbiology Department (ML) at Sahlgrenska University Hospital (SUH). These serum samples were used for comparative seroprevalence studies on the vaccine-preventable viruses MeV and RuV, and also as negative control for IgG reactivity to the mosquito-borne viruses CHIKV, ZIKV and WNV.

Additional samples of serum and CSF from Swedish TBEV-positive patients were retrieved from storage at -20^{o}C, in the ML at SUH.

Paper I

Data on IgG reactivity to MeV presented here were derived from serological analysis of 516 plasma and 215 serum samples from Rwandan and Swedish donors, respectively. Rwandan samples were collected according to the availability of measles ELISA kits, but also to regional representation in Rwanda. In addition, data on suspected cases of measles recorded from June 2010 to June 2011 were included. Results from analyses of MeV IgM (n= 544 plasma samples) and from MeV RT-PCR (n=31 nasopharyngeal swabs) were provided by the Rwandan national measles surveillance programme. WHO granted the permission to include data on measles vaccine coverage and the measles incidence reported by Rwanda and neighbouring countries for the period between 1980 and 2014.

Paper II

Seroprevalences of ZIKV and RuV IgG antibodies were tested on 874 plasma samples and 215 serum samples from Rwandan and Swedish donors, respectively. The anti-Zika IgG positive serum samples were analysed by real-time reverse transcription polymerase chain reaction (RT-qPCR) to detect ZIKV RNA.

Paper III

IgG to CHIKV and WNV were tested in 874 plasma and 199 sera from Rwandan and Swedish donors, respectively. Some Swedish samples with low volume were not tested. To investigate the probable cross-reactivity between WNV and TBEV, all samples that were positive for anti-WNV IgG antibodies among the Rwandan donors (n=91) and all of the samples from the 199 Swedish donors were tested for IgG reactivity to TBEV. Additional data included distribution of mosquito vectors, estimated from data on the 2012 assessment of yellow fever virus circulation in Rwanda.

Paper IV

In follow-up assessment of data derived from investigations of seroprevalence to CHIKV and WNV described in paper III, all sera from Swedish donors that were seropositive to WNV (n=28) were tested with the TBEV plaque reduction neutralization test (PRNT). However, Rwandan plasma samples could not be tested for TBEV neutralization activity with the PRNT for methodological reasons. Being a cell-based assay, the PRNT does not function with plasma samples due to the cell damage induced by the added anti-coagulants (EDTA). Additionally, serum samples with anti-TBEV IgM antibodies positive from Swedish patients (n=58) were also assayed for anti-WNV IgG antibodies.

For analysis of TBEV and WNV linear IgG epitopes on pepscan, samples were pooled as follow:

1. Pool 1 was prepared from 6 sera from confirmed Swedish TBE patients with high WNV IgG.
2. Pool 2 was prepared from 3 sera from Swedish blood donors with high WNV IgG results but negative in PRNT for TBEV antibodies.
3. Pool 3 contained 6 sera from Swedish blood donors with high WNV IgG and high TBEV IgG values, which also were positive for TBEV antibodies on PRNT.

4. Pool 4 included plasma from six Rwandan blood donors with high IgG results on WNV ELISA, as well as on TBEV ELISA.
5. Pool 5 was prepared from 4 CSF samples from Swedish patients with a confirmed diagnosis of TBE.

3.2 Analysis of samples

Serological and real-time RT-PCR studies of collected blood donor samples were carried out in the ML at SUH (Figure 8.2). Pepscan analysis of serum/plasma for IgG antibodies to linear epitopes of WNV and TBEV was carried out at PEPperPRINT GmbH, Heidelberg, Germany. The plaque reduction neutralization test for TBEV neutralizing antibodies was carried out at the Public Health Agency of Sweden, Stockholm.

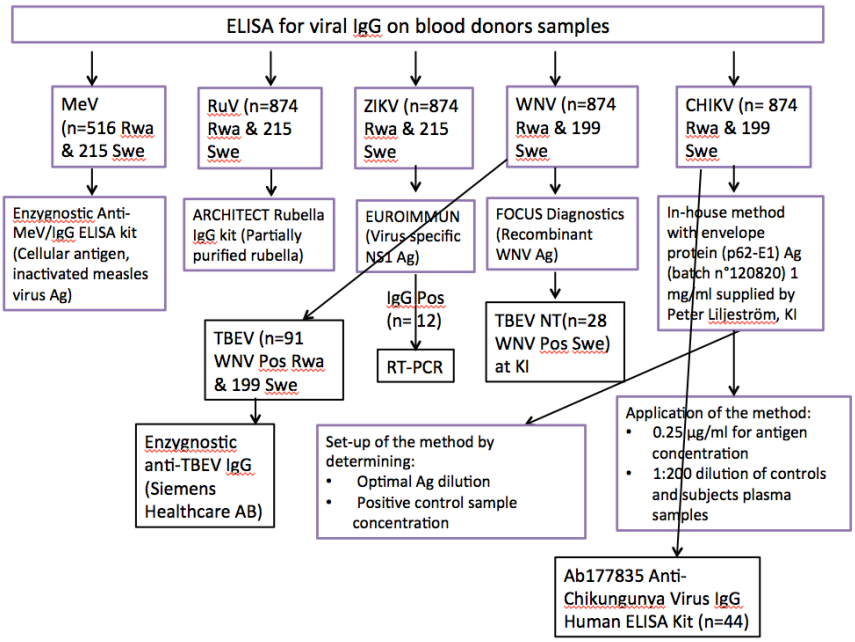

Figure 8.2 Analysis of Rwandan and Swedish blood donor's samples

3.2.1 Serological analysis

Seroprevalence results were based mainly on ELISA assay against the six RNA viruses tested, MeV, RuV, ZIKV, WNV, CHIKV and TBEV. Each reaction plate contained plasma samples from Rwandan participants as well as serum samples from Swedish blood donors or patients (Figure 8.2).

ELISA assay techniques are based on binding antibodies onto antigens coated on the wells of an ELISA microtiter plate. After rinsing, a secondary antibody conjugated to an enzyme is added, which binds to the specific antibody present on the antigen. Thereafter, the addition of a substrate produces a colour change, which occurs in the presence of the enzymatic activity of the bound conjugate. Positive and negative reactions, in relation to controls, are determined by optical density (OD) values obtained by the spectrophotometer (Figure 8.3).

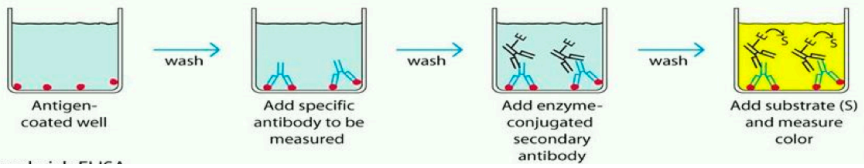

Figure 8.3 Serological testing techniques using indirect ELISA [178].

3.2.1.1 MeV ELISA for determination of IgG and IgM antibodies

To assess seroprevalence of MeV IgG antibodies in blood donor samples, sera and plasma were analysed using the Enzygnostic Anti-MeV/IgG Enzyme immunoassay kit (Siemens Healthcare Diagnostic Products GmbH, Marburg/Germany) according to the manufacturer's instructions.
Participants were divided into two groups, the first of those aged below 35 years and the second 35 years and above, to compare the seroprevalence before and after the introduction of the measles vaccine in Rwanda

Data on measles cases were derived from the Rwanda National registry on measles and rubella surveillance within the WHO global measles and rubella loboratory network. In Rwanda, measles cases were diagnosed using a commercial IgM ELISA kit from the same company as above, when serum samples were available for examination.

3.2.1.2 RuV and ZIKV IgG ELISA

IgG antibodies to RuV and ZIKV (targeting NS1) were measured using commercial ELISA kits provided by ARCHITECT Rubella IgG Abbott Sligo Ireland, and EUROIMMUN Medizinische Labordiagnostika AG Germany, respectively. The Rwandan and Swedish samples examined were analysed as described in the instructions provided by the manufacturers.

3.2.1.3 WNV and TBEV IgG ELISA

Anti-IgG antibodies to WNV were analysed using a complete commercial serological kit supplied by FOCUS Diagnostics (Cypress, California 90630 USA), according to instructions from the manufacturer.
Following the relatively unexpected high seroprevalence to WNV detected in Swedish blood donors, and the known problem of cross-reactivity between flaviviruses [179-183], we compared these WNV IgG findings in blood donors from Rwanda and Sweden with the seroprevalence of TBEV IgG in the two groups. To this end, we utilized the commercial Enzygnost Anti-TBEV IgG ELISA kit (Siemens Healthcare AB, Upplands Väsby, Sweden) to measure anti-TBEV IgG, according to the instructions from the manufacturer.

3.2.1.4 TBEV neutralization test

All Swedish sera testing positive for WNV IgG in the ELISA assay were tested, in the two dilutions 1/5 and 1/20, for cross-reactivity with TBEV, by plaque reduction neutralization test at the Public Health Agency of Sweden, Stockholm.

3.2.1.5 CHIKV IgG ELISA

The seroprevalence of IgG antibodies to CHIKV was assessed using an in-house viral recombinant envelope protein (p62-E1) Ag (batch no. 120820) 1 mg/ml (kindly supplied by Peter Liljeström, KI), and a two-phase analysis by ELISA was performed as described below.

Initially, we determined the optimal dilution of the CHIKV Ag solution, and of positive controls, and human plasma and serum samples. Different concentrations, by serial dilution of the antigen, were tested using as positive control a

serum from a cynomolgus macaque immunized with a DNA-launched CHIKV replicon encoding the CHIKV E protein, and boosted with recombinant modified vaccinia Ankara strain expressing the same antigen [115]. Horseradish peroxidase (HRP) mouse anti-monkey IgG conjugate was used as secondary antibody. The concentration of 0.25 µg/ml of the CHIKV antigen, a 1:200 dilution of the monkey positive control serum, and a 1:200 dilution of human plasma were found as optimal in order to provide the best signal/background ratio.

Thereafter, we assayed IgG seroprevalences using the CHIKV ELISA test on Rwandan and Swedish blood donor samples. Samples were screened in duplicate, at a dilution of 1:200. The positive control was a human plasma sample from a Mozambique patient who was found to be positive for CHIKV RNA on RT-PCR (sample provided generously by Dr Kerstin Falk from the Public Health Agency of Sweden, Stockholm). An HRP labeled anti-human IgG conjugate prepared in goat (Jackson ImmunoResearch Europe Ltd, UK) was used as secondary antibody.

Positive results were defined as values above the cut-off point determined by the first point of OD, which had the lowest number of samples (nadir), observed on a drawn chart based on the OD values of all samples (Supp. Figure Paper III). Thus, OD values greater than 0.45 were considered as positive.

We compared our findings from this novel assay using a serological commercial ELISA test (Ab177835 Anti-Chikungunya virus IgG human ELISA kit, Abcam, not intended for diagnostic use) on 9 strong positive, 9 intermediate positive, 9 weak positive, 8 negative CHIKV from Rwandan plasma samples, and on 9 Swedish CHIKV samples.

3.2.2 Real-time RT-PCR for Zika virus RNA

To investigate for a possible active infection of ZIKV, the ZIKV IgG positive samples (all collected from the Rwandan donors) were tested with reat-time RT-PCR at the ML at SUH.
Nucleic acids were extracted using the NucliSENS easyMAG instrument (bioMérieux, Marcy-l'Étoile, France) following the manufacturer's instructions. Thereafter, extracted nucleic acids were tested for ZIKV RNA by an in-house TaqMan real-time RT-PCR using specific primers:
(zikaFP1 5′-CACCAATTATGGACACCGAAG-3′,
zikaFP2 5′-CTCACCAATTATGGACACAGAAG-3′ and

zikaRP 5′-TCACCCAATCAAAGCCTGAG-3′)
and the BHQ1 labeled probe:
(zikaPCH 5′FAM-TCCAGGCTCTCTCTGGGACTTCC-3′-BHQ1) that target the relatively conserved NS3 region of the viral genome.

3.2.3 RT-PCR for measles virus RNA

Measles virus RNA was amplified in RT-PCR carried out at Uganda Virus Reseach Institute in Kampala, where the Qiagen OneStep RT-PCR kit was used with two primers targeting the 3' region of the nucleoprotein gene (measles genotyping kit v2.0 CDC, Atlanta):
forward primer (MeV216) 5'TGG AGC TAT GCC ATG GGA GT 3' and
reverse primer (MeV214) 5'TAA CAA TGA TGG AGG GTA GG 3'.
These target primers were previously described elsewhere [184].
The genotyping, based on nucleotide sequencing of the 634 base pairs product followed by BLAST homology analysis, was performed at Center for Vaccines and Immunology in Johannesburg, South Africa.

3.3 Statistical analysis

Data were recorded and analysed using the IBM SPSS Statistics vs. 20 (IBM Corp., Armonk, NY).
Seroprevalence to different viruses were presented as number (n) and percentage (%). Comparison of seroprevalences between Rwandan and Swedish donors was evaluated by the Pearson's Chi-squared test for independence, while the mean IgG OD values were compared using the Student's t-test for two independent samples. Significant results were defined by a p value less than 0.05.

3.4 Ethical considerations

This research project was reviewed and approved by the National Heath Research Committee (Ref: NHRC/2014/PROT/0196) of the RBC and the Ministry of Health in Kigali.
University Research Ethics committees at Kigali University Teaching Hospital (Ref: EC/CHUK/029/12, and Ref.: EC/CHUK/216/2016) and at Rwanda Mili-

tary Hospital (Ref: 002/010/2014) reviewed and cleared the study project. Sufficient information about the study was provided to all participants and those who accepted to sign the consent form were enrolled.

The Swedish blood donors were analysed anonymously and results could not be traced back to individuals. According to Swedish rules, ethical permission is not required in this context.

4. Results and Discussion

4.1 Paper I

This paper describes the seroprevalence of measles from 516 Rwandan and 215 Swedish blood donors, and outbreaks of measles infections during 2010-2011 in Rwanda. Finally, WHO data on vaccine coverage against measles in Rwanda from 1980-2014 in comparison to its neighbouring countries are included. This is the first seroprevalence study assessing the performance of the immunization program in Rwanda.

4.1.1 Measles seroprevalence

The seroprevalence to MeV was found to be 71.5% among Rwandan blood donors, as compared to 92.6% among the Swedish donors (table 8.1). Rwandan donors were divided in two age groups, those born after the introduction of the measles vaccine (in 1980), i.e. <35 years old and the older subjects born before 1980 (≥ 35 years old). The same age groups were valid for Swedish donors as well. When assessing the influence of age, younger Rwandans (< 35 years of age) showed a seroprevalence to MeV of 64.8% while their older counterparts presented a seroprevalence of 90.4%. Younger Swedish donors presented a slightly lower seroprevalence to measles of 88.9%, when compared to older Swedish subjects (≥ 35 years) who had a seroprevalence of 94.1%. As an indirect estimation of antibody quantities, the mean MeV IgG OD value was 0.68 from younger Rwandan donors, as compared to 1.2 from the older subjects. Similarly, younger Swedish donors showed a lower mean of MeV IgG OD value (0.69) when compared with their older counterparts, who had a mean OD value of 1.65. The most probable explanation of these findings was that the older subjects from both countries had been naturally infected, while a large proportion of the younger individuals showed immunity after vaccination only.

The lower measles seroprevalence among blood donors in Rwanda of 71.5% in comparison to the Swedish donors (92.6%), found in our study, might be influenced by the fact that only one dose of measles vaccine was administered in

Rwanda from 1980 until 2013, as compared to the two doses given in Sweden since 1982. Furthermore, Rwandan donors were younger in general (mean age: 29.1 years) in comparison to Swedish donors with a mean age of 44.3 years. The booster that was introduced in the Rwandan program of immunization as a second dose of measles vaccine from 2014 might improve both the seroprevalence and the strength of immunity as indicated by the OD values.

Table 8.1 Measles seroprevalence and mean age of Rwandan and Swedish donors

	Rwandan (n=516)			Swedish (n=215)		
	<35	≥35	Total	<35	≥35	Total
Positive	247 (64.8%)	122 (90.4%)	369 (71.5%)	56 (88.9%)	143 (94.1%)	199 (92.6%)
Negative	94 (24.7%)	10 (7.4%)	104 (20.2%)	4 (6.3%)	7 (4.6%)	11 (5.1%)
Equivocal	40 (10.5%)	3 (2.2%)	43 (8.3%)	3 (4.8%)	2 (1.3%)	5 (2.3%)
Mean MeV IgG OD	0.68 (0.62-0.74)	1.20 (1.1-1.3)	0.85 (0.79-0.91)	0.69 (0.58-0.80)	1.65 (1.54-1.76)	1.38 (1.28-1.48)
Mean age (Yr (range))		29.1 (18-62)			44.3 (18-75)	

4.1.2 Measles incidence

Out of 544 suspected cases of measles collected in Rwanda during the 2010-2011 surveillance of measles, 76 cases were confirmed by IgM positive results on ELISA (Table 8.2). Two age groups of these suspected cases were considered, based on the definition of pediatric (<16 years) and adult (≥ 16 years) age in Rwanda. Of the 544 suspected cases, 91.4% were < 16 years old, from whom 58 cases (11.7%) were IgM positive, while 8.6% were ≥ 16 years old with 18 cases (38.3%) being IgM positive. The higher proportion of confirmed measles cases within the older group might be explained by the possibility that a higher proportion of infections in young children were presenting clinical manifestations similar to those of measles, as compared to presentation in adults.
Of the seventy-six IgM positive measles cases, only 16 (21%) were aged ≥ 16 years old while 60 (79%) were < 16 years. This confirms that measles is still a childhood infection in Rwanda.

During the same measles surveillance period, 31 nasopharyngeal samples were collected from suspected cases, and 21 (67.7%) of these samples were PCR positive. Genotyping revealed that all measles positive samples from Rusizi and Nyarugenge districts were of the B3 subtype, and further phylogenetic analysis suggested that this strain might have been imported from DRC. Further support

for this hypothesis is the incidence map of patients positive for IgM against measles [185]. As shown there (paper I, figure 2), a large majority of positive subjects were found in regions bordering DRC and Burundi.

Table 8.2 Suspected and confirmed measles cases in Rwanda

	Suspected measles cases (n (%))	IgM-positive (n (%))	Positive PCR results	Genotype
Age				
<16 years	497 (91.4)	58 (76.3)*		
≥16 years	47 (8.6)	18 (23.7)**		
Total	544	76		
NPH samples	31		21 (67.7%)	B3

* Of the younger subjects 58/497 (11.7%) were IgM positive

** Of the older subjects 18/47 (38.3%) were IgM positive

As a possible explanation of the presently low incidence of measles cases in Rwanda despite our findings of a low seroprevalence, vaccine coverage has been kept high during the last decades and has also included immunization of refugees [186, 187]. The reported mean vaccine coverage rate was > 80% for the last 30 years (since 1988), except in 1994 (year of genocide) where everything was destroyed including healthcare facilities and human resources, and a record low coverage of < 25% was recorded [188-190]. The incidence of measles varied accordingly. Since 1988, measles incidence was below 10,000 cases, except during the war period where high incidence was recorded: 17,429 cases in 1992 and 28,874 cases in 1995 [188]. Even though the vaccine coverage is currently high, outbreaks of measles can still be anticipated in Rwanda in the future, given that almost 30% of the donors tested here were non-immune.

Compared to its neighbouring countries (table 8.3) during the period of 2003 to 2014, the reported measles vaccine coverage was high and the number of measles cases was low in Rwanda.
However, the few unvaccinated individuals and the non-responders to the vaccine in Rwanda might still be susceptible to imported measles [191, 192], as indigenous measles strains might be eradicated by the sustained high measles vaccine coverage [193, 194].

Table 8.3 Comparison of measles vaccine coverage and of measles cases in Rwanda and its bordering countries during 2003 to 2014, based on data from WHO. Mean values for the period are presented.

	Rwanda	Tanzania	Burundi	Uganda	DRC
Measles vaccine coverage (mean (%)) 2003-2014	93,4	94	89	74	67
Measles incidence, no. of cases (mean) 2003-2014	197	1827	184	4739	60910

The high number of measles cases reported by DRC and Uganda might be explained by their vast countries, in addition to their relatively low vaccine coverage rates. In Tanzania, the higher incidence of measles, despite similar vaccine coverage rate as Rwanda, may be explained (at least partly) by having a five times larger population. Interestingly, the DRC and Uganda are also improving their vaccination coverage rate with a mean of 89.8% and 92% respectively over the last 5 years (from 2013 to 2017) [188].

To conclude, the higher seroprevalence to MeV and the higher mean OD values to this virus observed in older donors as compared to the younger donors of both countries might be explained by the stronger immune response induced by natural infection as compared to that after vaccination. The finding that Swedish donors showed higher seroprevalence and higher OD values in comparison to their Rwandan counterparts is most likely explained by the use of only one dose of MeV vaccine during 1980-2013 in this country, and the temporary absence of a functioning immunization program during and after the genocide in 1994. Despite these setbacks, immunity to MeV in Rwanda has substantially improved during recent years, as shown by the decreasing incidence of measles in the country. In light of these data, the threat of new larger outbreaks of measles within the country seems relatively low at present, despite the low seroprevalence. However, the situation in neighbouring countries should be observed with caution.

4.2 Paper II

ZIKV and RuV seroprevalences among Rwandan and Swedish blood donors were investigated. In particular, Rwandan women of childbearing age were assessed for their susceptibility to these two viruses with the potency of causing fetal malformations through congenital infections. For RuV, the investigation of specific immunity was motivated by the recent introduction of general vaccination against this virus in Rwanda.

4.2.1 Seroprevalence of ZIKV

ZIKV IgG was detected in only 12 of the 874 Rwandan blood donors examined, resulting in a seroprevalence of 1.4 %. No Swedish donor was found to be positive for anti-ZIKV IgG antibodies.
Of these 12 anti-ZIKV IgG antibody positive Rwandan samples, 5 were derived from the Eastern Province, 4 from the Southern Province, 2 from Kigali City, and 1 from the Western Province. All these anti-ZIKV IgG positive plasma samples tested negative for RT-PCR targeting this virus, arguing against ongoing infection in any of the subjects.
From a total of 303 female Rwandan donors, 297 were of childbearing age (<45 years). As only two of them had anti-ZIKV IgG antibodies, almost all the females of childbearing age were seronegative and thus susceptible to ZIKV (295/297, 99.3%).

Recently, a low seroprevalence of ZIKV, 0.4%, was reported in healthy subjects from Ethiopia [195]. This low seroprevalence indicated an absence of herd immunity to this virus also in Ethiopia. Taken together, the two studies suggest that large populations of east Africans are susceptible to ZIKV, should new (or old) genotypes be introduced and start to circulate.
Since infection of pregnant women with this virus might spread to the fetus to cause congenital malformations including microcephaly [196], the high rate of seronegativity in Rwandan women of childbearing age to ZIKV is a reason for concern. Although the global spread of ZIKV seems to have diminished at present, preparedness for surveillance of this virus can be recommended.

All the twelve Zika IgG positive samples were found to be negative on RT-PCR. Here, a missed diagnosis due to inappropriate timing of sampling, such as collections being done during the late phase of the infection, could not be completely

ruled out [143]. However, a more probable explanation for this negative finding could be the absence of active infection in Rwanda during the study period. But it should also be mentioned that flaviviruses are not regularly tested for among febrile patients in Rwanda at present, and that most such cases are diagnosed as "presumptive malaria" which is endemic in our country. In this context, a recent serological study conducted in hospitalized patients in Uganda, a neighbouring country to Rwanda and the original site of isolation of ZIKV, indicated this virus as causing fever in singular cases[197]. However, confirmation by detection of viral RNA and/or genetic sequencing was not performed, thus further studies are needed to settle the question whether ZIKV is currently circulating in the region.

The low seroprevalence of ZIKV IgG observed in Rwanda is comparable to our findings in Swedish donors where no samples were seropositive to this virus. Despite the high abundance of mosquito vectors in Rwanda and its geographical location within a region endemic for flavivirus, our data argues against human spread of ZIKV in this country. However, the duration of immunity to this virus is largely unkown, and the half-life of anti-ZIKV IgG antibodies might be of short duration. If this is the case, i.e. that ZIKV does not confer a long-lasting protective immunity, a relatively recent circulation of this virus could not be ruled out.

Rwanda has been classified by the Centers for Disease Control (CDC) as a country with high risk of ZIKV spread [198]. Results from our study showing a very low seroprevalence to ZIKV among blood donors in Rwanda might support this classification [199]. However, absence of an infection in a country might also be interpreted as a low risk for spread. As an example, the classification of Rwanda by the WHO as a high-risk country for yellow fever virus before 2014 [200] was followed by a changed estimation to that of a low-risk country [201]. This change was based on a survey on "Risk assessment of yellow fever virus circulation in Rwanda" that was conducted in 2012 [202], and which showed low seroprevalence in humans and absence of the virus in mosquito vectors. Whatever the case, Rwandans travelling to ZIKV-endemic areas may presently be at high risk of infection, as almost all blood donors were found to be non-immune to this virus. Thus, pregnant women in Rwanda, as well as in Sweden, could be cautioned against travelling to countries where ZIKV is endemic and congenital infections have been documented.

4.2.2 Seroprevalence of Rubella virus

Seven samples from three females of childbearing age and four male Rwandan donors were not tested for anti-RuV IgG antibodies due to low sample volume.

The seroprevalence to RuV IgG, investigated on 869 plasma samples from Rwandan blood donors, was high (91.9%), and comparable to that in their Swedish counterparts (92.1%). Of the 300 Rwandan female donors, 89% tested positive to anti-RuV IgG antibodies. Of these, 294 were of childbearing age, from whom 31 (10.5%) were seronegative and thus susceptible to RuV.

The high seroprevalence to RuV in Rwanda could only be explained by natural immunity, since the Rubella-containing vaccine (RCV) was recently (2013, catch-up for 15 years olds) introduced in Rwanda [189]. In contrast, the even higher seroprevalence of RuV IgG in the Swedish donors may result from a combination of natural immunity and of antibodies induced by the vaccine. Most likely, natural infection induces a stronger antibody response as compared to vaccination, which might explain the increase in seroprevalence as well as of increased anti-RuV IgG OD values with age in the Swedish cohort. The finding that anti-RuV IgG OD values decreased with age in the Rwandan donors was probably caused by antibody decrement over time in this naturally infected population.

As we can deduce from data presented in Table 8.4, RuV is circulating in Rwanda but not in Sweden at present. Fortunately, CRS cases have been absent in both countries during recent years, probably explained by high levels of immunity in their respective female populations. However, the negative reporting of CRS cases as presented in table 8.4, adapted from WHO data [188], might question the quality of CRS diagnostics in Rwanda, but also in Sweden. Protective effects of vaccination against CRS cannot yet (2015) be anticipated in Rwanda.

Table 8.4 Incidence of CRS, Rubella cases and RCV (%) in Rwanda and in Sweden over the last decade (2008 to 2017). Source: WHO.

	Condition	2017	2016	2015	2014	2013	2012	2011	2010	2009	2008
Rwanda	CRS	0	0	0	-	-	5	-	-	-	-
Sweden	CRS	0	0	0	1	0	0	1	0	0	0
Rwanda	Rubella	22	15	1	15	50	172	62	36	34	35
Sweden	Rubella	0	0	0	0	0	50	4	3	1	0
Rwanda	RCV	99	95	99	97						
Sweden	RCV	97	97	98	97	97	97	96	97	97	96

The continuous circulation of RuV in Rwanda (Table 8.4), despite such high seroprevalence justifies the introduction of general vaccination against this virus in Rwanda. Another support for RuV vaccination would be our finding of a relatively high proportion of females among the non-immune donors in Rwanda. We found that 11% (33/300) of Rwandan females were seronegative to RuV as compared to only 6.5% (37/567) of the male donors, $p<0.02$. In addition, since the RuV antibody titers decreased with age among Rwandan subjects (Figure 2. B, paper II), women that become pregnant at advanced age [203] might be infected and thus expose fetuses, with CRS as an end result. This is especially valid, as the improved hygiene and living condition of Rwandans may interrupt the booster from natural infection. Taken together, these factors justify the necessity of introducing and maintaining RuV vaccine within the Extended Program of Immunization in Rwanda.

4.3 Paper III

Seroprevalence of anti-CHIKV and anti-WNV IgG antibodies were investigated by analysis of plasma samples from 874 Rwandan and on serum samples from 199 Swedish blood donors. Positive IgG results were related to the distribution of mosquito vectors, which were earlier identified in Rwanda. To assess for serological cross-reactivity between WNV and TBEV, IgG antibodies to the latter virus was also investigated.

4.3.1 Seroprevalence of CHIKV

Seroprevalence of IgG to CHIKV in Rwandan blood donors was found to be high (63%), but differently distributed among the provinces. The Eastern Province in Rwanda reported the highest seroprevalence, 86.7%. In addition, Rwandan donors > 26 years old showed a higher seroprevalence for CHIKV IgG (77%), as compared to the younger (≤ 25 years) subjects (51%). Swedish donors showed a low seroprevalence of 8.5%, with lower mean OD value to the CHIKV antigen (0.75 as compared to 1.5 of Rwandans) of the positive samples.

The high seroprevalence of 63%, observed here among Rwandan blood donors, may predict an important circulation of CHIKV in Rwanda. Elsewhere in Africa,

a high seroprevalence to CHIKV was also reported. In 2004, the Busia District of Kenya reported a seroprevalence of 59.9% for CHIKV. In another study, conducted between 2010 and 2012 in western Kenya, the CHIKV seroprevalence was even higher at 66.9% [204, 205]. To further support an actual circulation among humans in east Africa, CHIKV was also isolated in Kenyan patients, and mosquito vectors were investigated and found to be competent for CHIKV as well [206, 207]. In addition, CHIKV was the most common arbovirus identified in acute febrile Ugandan patients when samples collected during 2014-2017 were analysed [197].

The lack of reported clinical cases in Rwanda might be explained by the shortage of diagnostic means, the lack of the awareness of arboviruses by medical doctors and the population in general, and above all the syndromic diagnostic approach by which Chikungunya fever might be misdiagnosed as malaria, an endemic parasitic mosquito-borne infection in Rwanda. On the other hand, the high seroprevalence of CHIKV in Rwanda might be a result of serological cross-reactivity within closely related alphaviruses [208], which was not investigated here. Especially the ONNV, which was found to be prevalent within African countries (Figure 8.4) where CHIKV is also prevalent (Figure 8.5), including those bordering Rwanda, should be investigated in future arboviral serostudies.

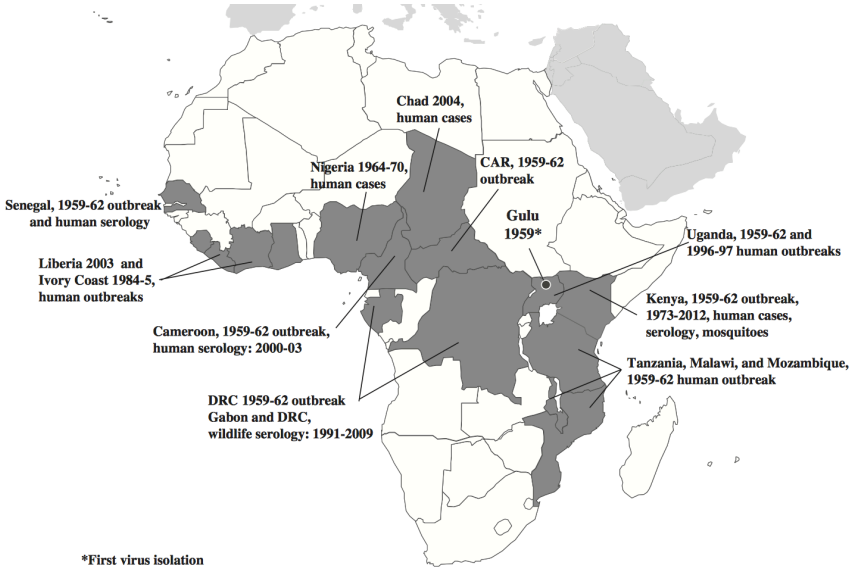

Figure 8.4 African map with countries where ONNV was isolated in human, mosquitoes, or other animals [209]. With permission from Taylor & Francis.

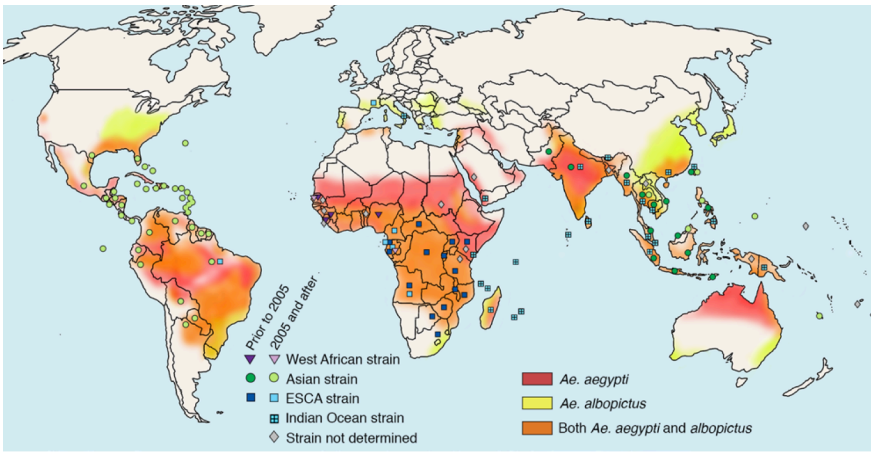

Figure 8.5 Geographical distribution of endemic CHIKV and its primary vectors [210].

As we were the first to use the p62-E1 antigen for anti-CHIKV IgG antibody investigation, we compared our results with a commercial test (Ab177835 anti-Chikungunya IgG, Abcam) on 35 selected Rwandan samples, based on the strength of IgG OD values from the in-house antigen assay, and on 9 Swedish samples with different results. Only our strongly positive samples from Rwanda were comparable between both tests: 7 out of 9 were positive, one sample was found equivocal and another negative. The remaining Rwandan samples, the intermediate and weakly positive, and the seronegative samples, in addition to all 9 Swedish samples, were found to be negative by the commercial test. We hypothesized that our in-house test might be more sensitive, allowing the detection of IgG reactivity also in more weakly positive samples, which may explain the high seroprevalence. This antigen was designed as a vaccine candidate, and therefore should be specific enough to induce immunity to CHIKV. On the other hand, the commercial test, which was aimed for research only and not for diagnostics, might have been designed to be less sensitive in order to avoid detecting cross-reactive samples. However, it should be pointed out that the performance of our new ELISA assay remains to be tested in febrile patients with suspected arboviral infection, in order to correlate results with the outcome of PCR analyses.

The seroprevalence for CHIKV IgG of 8.5% among Swedish donors could be explained by travelling to southern Europe where outbreaks of CHIKV are re-

ported [211-215], but also by possible cross-reactivity with Ockelbo virus, a Sindbis virus prevalent in Sweden [216-218].

The highest seroprevalence to CHIKV, which was found in the Eastern Province of Rwanda in our study, might be linked to reported circulation of CHIKV within its bordering countries, Tanzania and Uganda. This finding coincides with the highest burden of malaria reported from the Eastern Province of Rwanda, to which four out of the top five districts with most of malaria cases belong to [14].

The higher seroprevalence among older Rwandan donors compared to their younger counterparts and CHIKV IgG OD values that increase with age (Figure 8.6) might predict a continuous exposure to CHIKV with enhancement of the immune response over time.

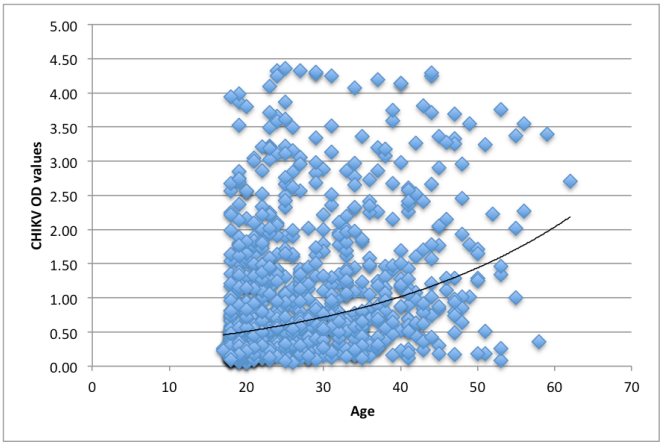

Figure 8.6 Distribution of CHIKV OD values with their trendline according to age of Rwandan blood donors.

4.3.2 Seroprevalence of WNV

Rwandan donors showed a seroprevalence to WNV of 10.4%, and again the Eastern region was found to exhibit the highest seroprevalent (33.3%) of all provinces in Rwanda. Suprisingly, Swedish donors had a seroprevalence to WNV of 14.1%.

Seroprevalences to WNV were reported earlier from several other African countries [219]. As an example, a low seroprevalence of 0.9% was reported from Ethiopia in the course of serostudies of yellow fever [195]. In Gabon, another study conducted in rural populations reported a higher seroprevalence of WNV IgG (27.2%), with local differences that could be related to the prevailing ecosystems. The lakes region recorded the highest seroprevalence for WNV of all regions, 64.9% [220]. The high seroprevalence for WNV observed within the Eastern Province of Rwanda coincides with the collection of the highest number of *Culex* mosquitoes (>35%) reported there, but also with the highest seroprevalence to CHIKV suggesting an important activity of arboviruses in that area. Of 73 samples showing dual positivity to WNV and CHIKV IgG among the Rwandan blood donors, 42 (57.5%) samples were from the Eastern Province. As discussed above, this province shares boundaries with Tanzania and Uganda where infections caused by those two viruses are frequently diagnosed.

The seroprevalence to WNV in Rwanda might also reflect serological cross-reactivity to other flaviviruses prevalent within the east central African region such as dengue fever or yellow fever [221, 222].
The relatively high seroprevalence to WNV seen in Swedish donors might be explained by an exposure to WNV locally or as tourists, especially in southern Europe, but above all, by cross-reactivity with TBEV, a flavivirus endemic in Sweden. To this end, we investigated TBEV seroreactivity in Rwandan (n=91) and Swedish (n=28) donor samples that were positive to anti-WNV IgG antibodies. Our results showed that 78.6% of Swedish donors were also positive for anti-TBEV IgG antibodies, with a higher mean OD value of 1.34, as compared to the mean OD value of 0.65 in samples from Rwandan donors who were seropositive to TBEV at a rate of 70.3%. Furthermore, the TBEV OD values of all the 91 Rwandan samples investigated were found to be lower than their corresponding WNV OD values. In contrast, 13 of the 28 Swedish donors had higher OD values against the TBEV antigen as compared to their corresponding OD values for the WNV ELISA, suggesting that almost half of the Swedish subjects that were positive for anti-WNV IgG antibodies primarily were seropositive to TBEV and showed a serological cross-reactivity to WNV. This possible dual

serological cross-reactivity between TBEV and WNV was further investigated by pepscan analysis in Paper IV.

4.4 Paper IV

In paper III, we documented a probable serological cross-reactivity between the two flaviviruses WNV and TBEV. The aims of Paper IV were to further challenge and elucidate this cross-reactivity. After finding a similar seroprevalence to WNV in Rwandan (10.4%) and Swedish (14.1%) blood donors in Paper III, we tested the hypothesis that the Swedish WNV seropositive donors were IgG-positive to TBEV rather than to WNV. This was the case, but somewhat surprisingly the majority of Rwandan WNV seropositive donors were also seropositive to TBEV, possibly suggesting a bi-directional serological cross-reactivity.

Here, we first performed a PRNT against TBEV on all 28 Swedish serum samples from donors positive to WNV. This test could only be performed on the Swedish samples, since the Rwandan samples consisted of plasma which had a probable toxic effect on the cultured cells used for the PRNT. In addition, we assayed for anti-WNV IgG antibodies in serum samples from 58 Swedish TBE-patients who had previously tested IgM positive to TBEV. Finally, we mapped, by pepscan, amino acids sequences of linear epitopes through overlapping peptides representing the glycoprotein E of WNV and of TBEV on four different pools of serum/plasma samples as defined in Materials and Methods, paper IV. Briefly, Swedish and Rwandan donors that were dually seropositive to WNV and TBEV were tested, with Swedish TBE patients included as controls. In addition, CSF samples that were IgG positive to TBEV were also pooled and included for epitope mapping. Lastly, amino acids sequences of linear epitope were determined by analysis of reactivity of the pools to all the peptides.

4.4.1 Dual seroreactivity of Swedish samples to WNV and TBEV, and PRNT for TBEV

Of 28 serum samples from Swedish donors that were anti-WNV IgG antibody positive, 22 (78.6%) were also positive for anti-TBEV IgG antibodies. Of these 22 dually seroreactive samples, 18 (81.8%) tested positive for PRNT-TBEV, indicating a past TBEV infection or vaccination against this virus. Most likely, their anti-TBEV antibodies were cross-reactive with WNV. Further support for the existence of such a reactivity came from our testing of 58 TBEV IgM positive samples from notified TBE patients, when 31 (53.4%) of these samples also tested positive for WNV IgG. Since infections with these two viruses are rare events in the Swedish population, it is unlikely that any of the TBE patients also had experienced an infection with WNV.

The high proportion of positive reactions in PRNT-TBEV from Swedish WNV seropositive donor samples could largely explain the surprisingly high seroprevalence to WNV found among Swedish blood donors. In 18/28 (64.3%) of these samples, anti-TBEV IgG antibodies were demonstrated. The one PRNT-TBEV positive sample, which was TBEV IgG negative, might be due to the high specificity of the PRNT compared to ELISA test. The high proportion of TBEV IgM positive samples from actual TBE patients that tested positive to WNV IgG underscores this cross-reactivity. Although PRNT could not be performed on the Rwandan samples, the finding of much lower OD values of anti-TBEV IgG antibodies from Rwandan samples seropositive to WNV suggests cross-reactivity from WNV to TBEV, i.e. the opposite direction of that found in the Swedish samples. Furthermore, this finding argued against circulation of TBEV in Rwanda.

4.4.2 Analysis of IgG reactivity to linear epitopes of the E protein of TBEV and WNV in Rwandan and Swedish samples

Human serum pool 1, which consisted of confirmed TBE patients from Sweden, showed a moderate response against the TBEV peptide QKGSSIGRVFQKTKK and a very low response against WNV peptides WRNRETLMEF. This pool was drawn during an early phase of TBE, since these samples also were IgM positive to TBEV. Our findings suggest that the linear TBE epitope QKGSSIGRVFQKTKK evokes early IgG reactivity.

Human serum pool 2, of Swedish donors who were WNV IgG positive but PRNT-TBEV negative, showed reactivity of diverse magnitudes against TBEV peptides, where again the sequence QKGSSIGRVFQKTKK had the highest fluorescent intensity, and against WNV peptides with DLTPVGRLVTVN as the main epitope. The results suggest dual immunity despite the negative TBEV serology in this pool, since the negative control showed less reactivity to both peptides.

Human serum pool 3, of Swedish donors with WNV IgG positive and NT-TBEV positive, showed a moderate response against both the TBEV peptide QKGSSIGRVFQKTKK and the WNV peptide DSCVTIMSKDKPTID. Most likely, this pool demonstrated dual immunity.

Human plasma pool 4, of Rwandan donors seropositive to both WNV and TBEV, showed the strongest and the most complex response in the pepscan analysis when compared to the Swedish pooled samples. Surprisingly, the Rwandan pooled sample showed a moderate to strong IgG response against TBEV peptides with the consensus motifs IVYTVKVEPHT, NETHSGRK, RKTASFTISSEKTI, AQNWNNAERLV, SGHVTCEVGLEK and SGHDTVVMEV, as compared to a weak to moderate IgG response against WNV peptides SCVTIMSKDKPTID, IKYEVAIFVHGPTTV, GTKTFLVHREWFMD, WRNRETLMEF, ALAGTIPVE and LFLSVNVHA. In addition to strengthening the serological finding of their WNV exposure, these results suggest that Rwandan donors could have been exposed to hitherto unknown TBEV-like viruses.

The human CSF pool 5 from confirmed TBE patients exhibited a weak but clear response against two TBEV peptides with the consensus motifs WQVHRDWFN and GAQNWNNAERLVE. Interestingly, the corresponding serum pool 1 showed almost no reactivity to these peptides, arguing for a selective (or earlier) exposure of these linear epitopes within the CNS. There were no visible respons-

es from the CSF pool against any peptides of the WNV variant of glycoprotein E.

In summary, the three Swedish serum pools showed some similarities in reaction to linear peptides of both WNV and TBEV (Figure 8.7). Their fluorescence intensity was higher for peptides representing TBEV as compared to WNV, except for the pool 2, seronegative to TBEV, where the fluorescence intensity was slightly higher for WNV. There were no obvious sequence similarities among the epitopes defined above, from glycoprotein E of WNV and TBEV, that could explain the serological cross-reactivity that we found by ELISA tests against these two viruses in paper III. Although detailed documentation of the antigens of these two commercial tests is lacking, they most likely contain, in addition to their respective E proteins, several other viral and cellular proteins that could influence cross-reactivity.

The CSF pool from the Swedish TBE patients showed weak to moderate response against two TBEV epitopes only. These peptides represent the first linear and intrathecal TBEV-epitopes described, and they might well be specific and could therefore serve to distinguish anti-TBEV IgG from antibodies reactive with other flaviviruses, in the future. The other main finding was that Rwandan plasma samples reacted with several TBE-peptides of which some were conserved across the hitherto sequenced members of the sub-family of tick-borne flaviviruses. Such viruses should be sought for in Rwandan patients with CNS infections.

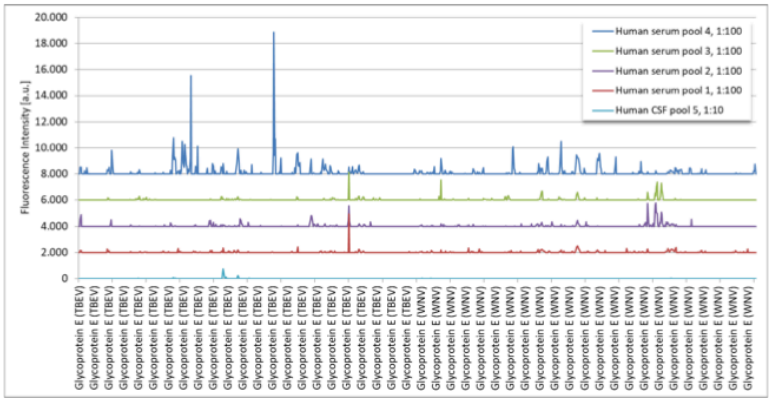

Figure 8.7 Comparison of the antibody responses of human serum pools 1, 2, 3 and 4 and CSF pool 5. The strongest response was found for serum pool 4 against epitopes of TBEV and WNV glycoprotein E variants, while CSF pool 5 only responded against epitopes of TBEV.

5. Conclusions

We have, for the first time in Rwanda, conducted seroepidemiological studies of two vaccine-preventable viruses, MeV and RuV. The seroprevalence of MeV among Rwandan blood donors was low, probably as a result of only one dose of MeV vaccine being provided until 2013. This susceptibility to MeV in large parts of the population may lead to future outbreaks of measles, especially among Rwandan subjects of young age (<35 years old) who had the lowest seroprevalence (64.8%). Most likely, this risk situation will be improved by the recent introduction of the second dose of MeV vaccine. In addition, the high MeV vaccine coverage rate (>90%) and the limited and decreasing number of measles cases reported in Rwanda are indicators of a well functioning immunization program. Furthermore, the MeV strains detected in Rwanda during the 2010-2011 outbreak may have been imported, as the B3 genotype found by sequencing were suggested to originate from the DRC.

In general, the seroprevalence for RuV among Rwandan donors was high and reached levels comparable to that of Swedish blood donors. However, unlike the findings in samples from Swedish donors, antibodies to RuV were decreasing with age among Rwandan donors, who only had been naturally infected. A relatively high number of Rwandan female donors of childbearing age were found to be seronegative to RuV (10.5%), which indicated an increased risk of developing congenital infection with possible CRS. In addition to the recently introduced general immunization against RuV, a vaccination campaign specifically targeting this group seems warranted.

In the same population of donors, we also assessed the immunity to three mosquito-borne viruses in Rwanda: ZIKV, CHIKV and WNV. Most Rwandan blood donors were seronegative to ZIKV, and therefore susceptible to infection with this virus. Especially females of childbearing age are at risk should the virus start to circulate in Rwanda. The ZIKV IgG positive samples were negative on ZIKV RT-PCR, thus arguing against a current circulation of ZIKV in Rwanda. However, the identification of mosquito vectors of ZIKV in Rwanda is an observadum, and a preparedness plan of preventive measures can be recommended.

In contrast, Rwandan blood donors showed a high seroprevalence to CHIKV, which increased with age, suggesting an extensive and continuous circulation of this virus in Rwanda. We recommend that infection with CHIKV should be considered among several differential diagnoses in febrile patients. In this context, our new serological test could be useful. Though a low seroprevalence to WNV was reported from Rwandan donors, the highest seroprevalence (>33%) found within the Eastern Province indicated the importance of considering also WNV while managing febrile patients from this region. Furthermore, its vector, *Culex,* was the most abundant mosquito species identified in that area. In addition, the dual seropositivity to CHIKV and WNV seen among Rwandan donors from the eastern region, and the relative abundance of *Aedes* and *Culex* mosquitoes suggested an important co-circulation of these two arboviruses in Rwanda. Swedish donors presented a low seroprevalence of CHIKV IgG, probably due to a limited exposure.

Lastly, we found a possible serological cross-reactivity between the two flaviviruses WNV and TBEV. This was suspected due to a relatively high seroprevalence of WNV IgG in Sweden (14.1%) as compared to Rwanda (10.4%). Surprisingly, a large majority of the WNV seropositive donors from both countries were reactive also in the TBEV test. Furthermore, in Rwandan blood donors we found a reactivity to several TBEV-related linear epitopes, which seemed completely conserved within the tick-borne flaviviruses, suggesting a circulation of unknown tick-borne flaviviruses in Rwanda. To conclude, future diagnostic study of febrile patients should include sequencing studies that may contribute to the discovery of such viruses, and improve our understanding of the febrile patient' syndrome.

6. Future Perspectives

The seroprevalence studies of MeV and RuV in this thesis could serve as a baseline for future evaluations of the general vaccination program in Rwanda. The low seroprevalence of MeV, with immunity provided by natural infection or by one dose of MeV vaccine, raises a question regarding MeV vaccine efficacy in relation to the MeV strains circulating in Rwanda. Thus, the effect of the second dose of MeV vaccine, introduced in 2014, should be serologically investigated and compared to our baseline results. We anticipate that the switch from one dose to two doses of the vaccine will greatly improve the here documented low seroprevalence for MeV in Rwanda over time. But such an expected improvement could be counteracted by a low circulation of MeV in the future, since the effect of natural boosting will diminish. The external pressure from neighbouring states as regards MeV will most likely continue for some time, why serosurveillance of immunity to this virus should be continued in Rwanda.

In the same context, the high seroprevalence for RuV, that however decreases with age, is presently related to natural infection only. Our finding of seronegativity in a relatively large proportion of women of childbearing age should be followed up by vaccination, for example at maternal and child health (MCH) clinics. Although catch-up vaccination of teenagers against RuV has been carried out in Rwanda, in addition to the recently introduced national RuV immunization program of children, serotesting during pregnancy and vaccination of IgG-negative subjects could be recommended.

The seroepidemiology for ZIKV in Rwanda was, in essence, negative. This is a strong indication against a spread of ZIKV in Rwanda, which should be beneficial from a general health aspect. On the other hand, this finding indicates a risk of future spread of the virus, since the population is non-immune. We recommend a continued awareness of the dynamic spread of ZIKV globally, not least as regards introduction of Pacific-American strains into Africa. To this end, improved diagnostics could be developed, including ZIKV and RuV among the microbial agents sought for, when investigating suspected congenital infections and/or malformations in Rwanda.

As a consequence of the here described high seroprevalence of CHIKV in Rwanda, febrile patients should be screened for CHIKV RNA, but also for other medically important arboviruses such as Dengue, O'nyong-nyong, and Rift Valley fever viruses which are frequently reported from this region of Africa. The introduction of such PCR tests will improve the management of patients who presently are classified as having "presumed malaria". Likewise, the screening of WNV RNA from febrile patients with/without meningo-encephalitis may be also justified, at least in the Eastern Province with regards to the reported seroprevalence and the abundance of *Culex* mosquitoes in this part of Rwanda.

The probable serological cross-reactivity between viruses of the same families such as between ONNV with CHIKV, or Dengue virus with WNV, but also of other genetically related arboviruses that share the same vector should be investigated. This is certainly valid because of the high seroprevalence shown for CHIKV and WNV, and the highest abundance of *Culex* and *Aedes* mosquitoes found in the Eastern Province of Rwanda. Finally, the reactivity to a TBE-related linear epitope found in Rwandan blood donors raise the suspicion of the presence of unknown tick-borne flavivirus(es) in Rwanda, which should be investigated for. To this end, there is a need also for investigating the prevalence of different species of ticks in Rwanda, in addition to *Culex* and *Aedes* mosquitoes, and their respective vector competency for transmission of arboviruses.

In summary, this thesis has contributed to the diagnostic development and implementation within the field of clinical virology in Rwanda. We anticipate that such development will contribute to the combat of medically important viruses, which may improve the general health of the population, not least for children and their mothers.

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