IMMUNOGENICITY STUDIES OF THE YELLOW FEVER VACCINE IN HEALTHY INFANTS AND ESTABLISHMENT OF SEROLOGICAL DIAGNOSTIC TOOLS FOR THE DETECTION OF YELLOW FEVER VIRUS INFECTIONS

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Declaration of Authorship

I hereby certify that the thesis presented here is my own work and was written without literature other than the sources indicated in the bibliography. Information used from the published or unpublished work of others has been acknowledged in the text and has been explicitly referred to in the given list of references. The present work has not been submitted, either in part or completely, for a degree at this or any other University.

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Abstract

Yellow fever (YF), an acute viral hemorrhagic fever caused by the yellow fever virus (YFV), is still one of the most feared diseases. YFV is a positive sense single-stranded enveloped RNA virus that belongs to the group of *Flaviviruses* and is transmitted to humans through the bite of infected mosquitoes. Primary endemic regions for YFV are sub-Saharan Africa and South America. YF counts as one of the neglected tropical diseases due to recurring outbreaks in the above-mentioned regions despite the availability of a safe and efficacious vaccine, i.e. YF-17D.

The focus of this dissertation lay on the investigation of the efficacy of the YF-17D vaccine in infants and the development of two different serological assays for the detection of YFV infections. This resulted in three projects, of which the first project aimed at analyzing immunogenicity of the YF-17D vaccine in infants when administered concomitantly with the measles vaccine and a new meningococcal A conjugate vaccine (MenAfriVac, PsA-TT). The two clinical studies were conducted in Ghana and Mali among infants who received the meningococcal A conjugate vaccine (PsA-TT) concomitantly with Measles and YF vaccines at 9 months of age. YF neutralizing antibody titers were measured using a microneutralization assay. In both studies, the meningococcal A conjugate vaccine did not adversely affect the immune response to the concomitantly administered YF vaccine at the age of 9 months. The magnitude of the immune response was different between the two studies, with higher seroconversion and seroprotection rates found in Mali when compared to Ghana. Therefore, further studies are warranted to better understand the determinants of the immune response to YF vaccine in infancy.

In order to further investigate these serum samples, the second project dealt with the establishment of an indirect enzyme-linked immunosorbent assay (ELISA) for the detection of YF IgG and IgM antibodies. Till date the gold standard in serodiagnosis is the plaque reduction neutralization assay. This assay however, is time consuming and laborious. Therefore, rapid and specific diagnostic tests for the serological analysis of serum samples are of immense importance - especially when considering the increasing numbers of yellow fever cases in recent years. The newly established indirect YF IgG/IgM ELISA would allow a much faster and easier analysis of multiple serum samples at the same time. An indirect ELISA based on the recombinant NS1 protein was successfully developed. With this newly established indirect ELISA 190 serum samples, which have been obtained from the above mentioned clinical studies, were analyzed in regard to IgM and IgG antibodies against YFV.

In addition, a third project was defined, which dealt with the execution of different virological methods used in YFV diagnosis as well as the evaluation of the subsequent results on a new system for real time analysis of cell proliferation with cell impedance measurements (Roche xCELLigence™ system). Therefore, standard virological YFV protocols have been transferred and established on to this novel system. Furthermore, two different published mathematical models for the quantification of YF neutralizing antibodies in serum samples have been compared. All the standard virological methods used in YFV diagnosis could be successfully transferred on to this new system. Moreover, both neutralizing antibody determination strategies could be applied on serum samples with known YFV neutralizing antibody titers. However, both strategies show weaknesses due to which further testing with more samples is required.

Zusammenfassung

Gelbfieber (GF) gehört aktuell zu den am meisten gefürchteten, durch Mücken übertragenen Krankheiten. Ursache dieser Erkrankung ist das Gelbfiebervirus (GFV), welches endemisch in Subsahara-Afrika und in Süd-Amerika auftritt. GF gilt als vernachlässigte tropische Krankheit, denn obwohl es den sicheren und effizienten Impfstoff GF-17D gibt, treten wiederholt zahlreiche Ausbrüche in den oben genannten Regionen auf.

Diese Dissertation beschäftigt sich mit der Untersuchung der Effizienz des GF-17D Impfstoffs in Säuglingen und der Entwicklung zweier serologischer Tests zur Detektion von GFV Infektionen. Hieraus ergaben sich drei Projekte, wobei sich das erste Projekt mit der Untersuchung der Immunogenität des GF-17D Impfstoffs in Säuglingen beschäftigt, wenn dieses zusammen mit dem Masern- und einem neuen Meningokokken - Meningitis A - Konjugat Impfstoff (PsA-TT, MenAfriVac) verabreicht wird. Grundlage waren zwei klinische Studien, wobei 190 neun Monate alte Säuglinge in Ghana und Mali gleichzeitig die Meningokokken-Meningitis A (PsA-TT), Masern- und GF-Impfungen erhielten. GF-neutralisierende Antikörper wurden mittels eines Mikroneutralisationstests detektiert. In beiden Studien konnte gezeigt werden, dass bei einer gleichzeitigen Verabreichung die Immunantwort des GF-17D Impfstoffs in 9 Monate alten Säuglingen nicht durch den PsA-TT Impfstoff beeinträchtigt wurde. Die Intensität der Immunantwort war zwischen den beiden Studien unterschiedlich. Serokonversions- und Seroprotektionsraten waren in Mali höher als in Ghana. Dies macht weitere Studien notwendig, um die genauen Determinanten der Immunantwort nach einer GF-Impfung im Kindesalter zu verstehen.

Das zweite Projekt fokussierte sich auf die Etablierung eines indirekten Enzym-linked Immunosorbent Assay (ELISA) zur Detektion von GF-IgG und -IgM Antikörpern in den beschriebenen Seren von 9 Monate alten Säuglingen. Bis heute ist der Plaque Reduktions-Neutralisationstest (PRNT) der Goldstandard der Serodiagnostik, welcher jedoch sehr zeitaufwendig und mühsam in der Ausführung ist. Schnelle, spezifische Tests zur serologischen Untersuchung von Serum-Proben sind von enormer Wichtigkeit, insbesondere angesichts der steigenden Zahlen an GF-Erkrankungen in den letzten Jahren. Wir konnten erfolgreich einen indirekten ELISA entwickeln und etablieren. Dieser neu etablierte indirekte ELISA basiert auf dem rekombinanten NS1 Protein und erlaubt eine deutlich schnellere und leichtere Analyse von multiplen Serum-Proben. Die 190 Serum-Proben aus den oben beschriebenen Studien konnten erfolgreich mit dem neu etablierten ELISA auf GF-IgG und -IgM Antikörper analysiert werden.

Zusätzlich wurde ein drittes Projekt formuliert, welches sich mit der Ausführung von verschiedenen virologischen Methoden der GFV Diagnostik sowie der Auswertung von den daraus resultierenden Ergebnissen auf einem neuartigen System zur Echtzeit-Analyse der Zellproliferation mit Impedanz-Messung (Roche xCELLigence™ system) beschäftigt. Dafür wurden standardisierte virologische Protokolle auf dieses neuartige System übertragen und unter Berücksichtigung der dort geltenden spezifischen Anforderungen etabliert. Zusätzlich wurden zwei publizierte mathematische Modelle zur Quantifizierung von GF neutralisierenden Antikörpern miteinander verglichen. Ergebnis dieses Teilprojektes war die erfolgreiche Übertragung aller Standard-Protokolle der GF Diagnostik auf das xCELLigence™-System und eine erste Untersuchung einer geringen Anzahl von Seren. Beide mathematischen Modelle konnten zur Quantifizierung von neutralisierenden Antikörpern gegen das GFV angewandt werden. Dies ermöglicht nach weiterer Optimierung der Parameter eine neuartige und schnelle Analyse weiterer Proben aus zukünftigen Studien.

CONTENTS

Abbreviation

aa	Amino acid
APS	Ammonium peroxodisulfate
Aqua dest.	Water (distilled by Millipore)
Aqua mol.	Water (molecular grade)
ATCC	American Tissue and Cell Culture Collection
bp	Base pair
BSA	Bovine serum albumin
cDNA	Complementary (copy) DNA
CDC	Center for Disease Control and Prevention
CI	Confidence Interval
CI_x	Cell Index
CI_{NT50}	CI value reflecting 50% virus neutralization
CIT50	50% decrease of CI of the lowest serum dilution
CMC	Carboxymethyl-cellulose sodium salt
CPE	Cytopathic effect
CT T	Threshold cycle
C-terminus	Carboxyl-terminus
°C	Degree Celsius
C protein	Capsid protein
CO_2	Carbon dioxide
d	Day
ddNTP	Didesoxynucleoside triphosphate
dNTP	Desoxynucleoside triphosphate
DMEM	Dulbecco´s Modified Eagles Medium
DMSO	Dimethylsulfoxid
DNA	Desoxyribonucleic acid
ds	Double stranded
DTT	Dithiothreolin
E. coli	*Escherichia coli*
e.g.	Exempli gratia (for example)

E protein	Envelope protein
ECACC	European Collection of Cell Cultures
ECL	Enhanced chemiluminescent
EDTA	Ethylendiaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPI	Expanded Programme on Immunization
ER	Endoplasmatic Reticulum
FCS	Fetal calf serum
FNV	French Neurotropic Vaccine Strain
Fig.	Figure
g	Gramm
(x) g	Centrifugal force (9.81 m/s^2)
GFP	Green fluorescent protein
GMT	Geometric mean titer
h	Hour(s)
His	Histidin
HRPO	Horseradish peroxidase
i.e.	Id est (that is)
IFA	Immunofluorescence assay
IgG	Immunglobulin G
IgM	Immunglobulin M
kb	Kilo base
kDa	Kilo Dalton
L	Liter
LB medium	Luria Bertani Medium
M	Molar
M protein	Membrane protein
mA	Milli ampère
MAC	Membrane Attack Complex
min	Minute(s)
mL	Milliliter
mM	Millimolar

MOI	Multiplicity of infection
mRNA	Messenger RNA
MVP	Meningitis Vaccine Project
µg	Microgram
µL	Microliter
n	Amount
n.d.	Not done
nm	Nanometer
NSp	Non-structural protein
NT	Neutralization titer
nt	Nucleotide
N-terminus	Amino-terminus
O_2	Oxygen
OD	Optical density
PATH	Program for Appropriate Technology in Health
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PFU	Plaque forming units
pH	Potentia hydrogenii (concentration of hydrogen ions in solutions)
PRNT	Plaque reduction neutralization assay
PsA-TT	Meningococcal A conjugate vaccine
PS	Pig kidney epithelial cells
PVDF	Polyvinylidenefluoride
$qRTNT_{50}$	Quantitative real-time titer reflecting 50% virus neutralization
RF	Rheumatoid factor
RKI	Robert Koch-Institut
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT	Reverse transcriptase
RTCA	Real-time cell analyzer
$RTCAT_{50}$	Real-time cell analysis titer reflecting 50% virus neutralization

sec	Second(s)
SAP	Shrimp alkaline phosphatase
SD	Standard deviation
SDS	Sodium dodecyl sulphate
S.O.B.	Super optimal broth
S.O.C.	Super optimal broth with catabolite repression (by adding glucose)
TAE buffer	Tris acetate EDTA buffer
TEMED	Tetramethylethylenediamin
$TCID_{50}$	Tissue culture infectious dose reflecting 50% virus neutralization
TBE buffer	Tris borate EDTA buffer
TBE	Tick-borne encephalitis
TE buffer	Tris EDTA buffer
TM	Transmembrane domain
Tm	Melting temperature
TMB	3,3',5,5'-Tetramethylbenzidine
UTR	Untranslated region
V	Volts
WHO	World Health Organization
WNV	West Nil Virus
YF	Yellow Fever
YFV	Yellow Fever Virus
YFV-17D	life attenuated vaccine strain against yellow fever virus

1 Introduction

The first records about a yellow fever (YF) outbreak are from the Yucatan peninsula in 1648, in which this disease was described as black vomit, the characteristic nature of yellow fever [7]. The term yellow fever appears for the first time in the book *Natural History of Barbados* by Griffin Hughes in 1750. Yellow fever virus (YFV) originated in Africa and spread to the New World by trading ships. As trade gained more and more importance yellow fever broke out in major cities in America, Africa and in the islands in-between. In the time between 1668 and 1870 devastating epidemics raged in cities like New York, Philadelphia, Memphis and Charleston, but also in Europe claiming about 5,000 - 24,000 deaths and economic losses of almost $200 million [8]. The devastating epidemics caused widespread panic as the reasons for the disease were unknown, and its symptoms not so different from plague, another dreaded disease of the time. In 1881 the scientist Carlos Finlay put up the theory that mosquito bites could be the cause for spreading the disease. However, his theory could not be proved until 1900, when Walter Reed and his colleagues used human volunteers to prove that the mosquito vector, *Aedes aegypti,* was the critical factor in the dissemination of the disease [9]. Furthermore, they could demonstrate that the disease was caused by a filterable agent found in the blood of infected individuals. This finding led to the establishment of a Yellow Fever Commission by the International Health Board in 1915. The establishment of this commission was the first step for combating YFV and the mosquito vector, respectively. The aim of this commission was to eliminate breeding places for *Aedes aegypti* in areas where yellow fever was prevalent. Though the elimination was highly effective, the disease still remained in some places [9]. In the mid-1930s scientists discovered that the natural reservoir of the virus were monkeys and that the infection was spread between them through various mosquitoes. From time to time the virus was also transmitted from infected monkeys to humans by different vectors. The contact of these people with larger human populations in urban areas led to the development of epidemics, in which the virus was then transmitted by *Aedes aegypti* from person to person [10].

In 1927, the virus could be isolated for the first time by Adrian Stokes from a sick Ghanaian man, named Asibi. Using this strain, the so called Asibi strain, Max Theiler was the first to develop the live, attenuated 17D vaccine by passaging the virus for more than 200 times in cell cultures. In parallel to this another vaccine was developed from a wild-type strain, namely the French neurotropic vaccine strain (FNV). Whereas the 17D vaccine has been widely

acknowledged as one of the most effective and safe vaccines in use, the trade of FNV was prohibited, as it caused severe post vaccinal encephalitis [5].

Since then the progress in the research of YFV has been immense. It has been classified to the family of the *Flaviviridae* [11]. Members of the family share similarities in regard to their morphology, genome organization and replication strategy, but differ in their biological properties [11]. The availability of different tests simplified the identification and the detection of the virus [12]. However, till date there are still gaps in the understanding of the exact genetic mechanisms of members of this family. Moreover, the viruses are brought to public attention regularly as they consist of many human and animal pathogens causing outbreaks.

The YF outbreak in Angola and DRC in 2016 and its spread to neighboring countries as well as the most recent outbreak in Brazil in 2017 show the immense need of ongoing research and warrant intensified national action and enhanced international support.

1.1 Thesis Outline

This thesis is organized in four chapters. After an introduction into the topic of the YFV in chapter one, the thesis begins with the methods, which were employed during the work of the thesis in chapter two. The third chapter deals with the results, which have been obtained during this PhD work. These are presented according to the projects in three subchapters. The thesis is concluded in the fourth chapter by discussing the obtained results and giving an outlook on further steps for the future.

1.2 Yellow fever virus

YFV is the prototype member of the family *Flaviviridae*, which are divided into three genera: *Flavivirus, Pestivirus* and *Hepatitis C-Virus.* The genus *Flavivirus* contains approximately 77 viruses, among which are viruses causing serious diseases like the yellow fever, dengue fever, West Nile fever, Japanese encephalitis and tick-borne encephalitis. They are transmitted by arthropods and hence also counted among *arboviruses*. The name “flavus” originated from the Latin and means “yellow”. This name was given to the disease as one of the symptoms of yellow fever is jaundice [11].

Yellow fever is a major public health problem in endemic regions of Africa and South America. At present the disease still affects around 200,000 persons annually with 30,000 deaths per year, in spite of the availability of an effective, life-attenuated vaccine strain (YFV-17D) [13, 14].

With the rise in air travel the risk of introducing and spreading yellow fever to North and Central America, the Caribbean and Asia increases. Control strategies for this disease include vaccination of people living in endemic regions and elimination of mosquito populations by destroying mosquito-breeding sites as the virus is transmitted through the mosquito vector, *Aedes aegypti*. In this way *Aedes aegypti* populations were eliminated from several countries resulting in a significant reduction of mosquito-borne viral diseases, like dengue and yellow fever [15]. Although this program had been successful initially, it was not continued which led to the re-emergence and widespread distribution of diseases caused by mosquitoes. The *Aedes aegypti* populations have returned and with them yellow fever and dengue [16]. Till date, there is still no cure and the only treatment available is for symptomatic support. Therefore, rapid diagnosis of the disease and corresponding diagnosis tests to assist in patient management are one of the major concerns of public healthcare institutions [12].

1.3 Morphology and Genome

Little is known about the structural features of the yellow fever virus. However, for some Flaviviruses like West Nile (WN), Japanese encephalitis (JE), tick-borne encephalitis (TBE) and dengue structures have been analyzed by cryo-electron microscopy and X-ray crystallography [17-22]. Therefore, due to the similarity of the molecular structures and antigenic relationships amongst Flaviviruses it can be assumed that the above-mentioned findings regarding structural organization and properties also apply for YFV particles.

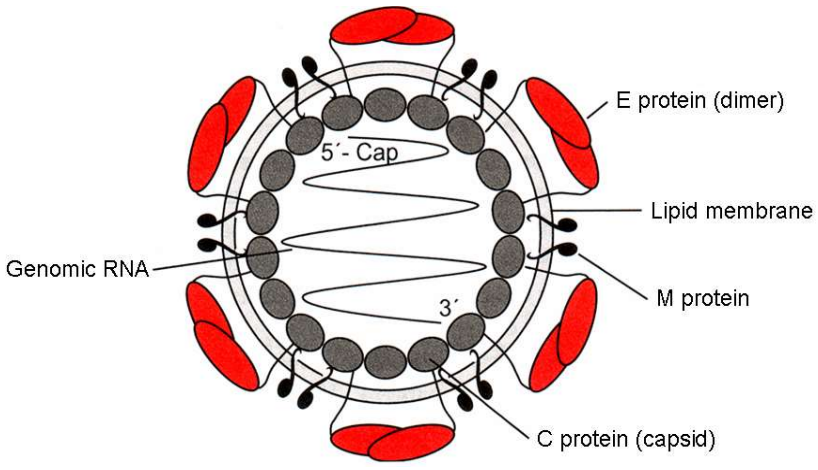


Fig. 1 Morphology of the YFV virion
Flaviviruses are surrounded by a lipid membrane and a capsid. The outer layer, which is the lipid membrane, consists of the glycosylated envelope proteins (E proteins) and the membrane proteins (M proteins) and the capsid is composed of the C protein, which forms the nucleocapsid and embraces the genomic RNA [1].

The morphology of the YFV is shown in Fig. 1. The Flaviviruses possess a diameter of 40-50 nm. An envelope surrounds the nucleocapsid, which has a diameter of 25-30 nm. The nucleocapsid consists of the non-glycosylated C protein (capsid), which embraces the viral genome and in parts is associated with it. The outer envelope is composed of a lipid bilayer and contains two or more species of glycosylated E proteins (envelope) and non-glycosylated prM proteins (membrane) [**Fig. 1**][11].

The genome of the Flaviviruses consists of a single, positive stranded RNA of approximately 11 kb (10862 base pairs for YFV) and contains only one open reading frame as can be seen in Fig. 2. This positive stranded RNA is capped at its 5'end, and serves as mRNA for the translation of only one polyprotein. The 3'end on the other hand is not polyadenylated, but is rich in adenosines. Both ends contain regions, which are not translated [**Fig. 2**] [1].

The polyprotein codes for 10 proteins – three structural proteins and seven non-structural proteins (NS). These are processed co- and post-translationally by multiple host proteinases and by a viral serine proteases [11].

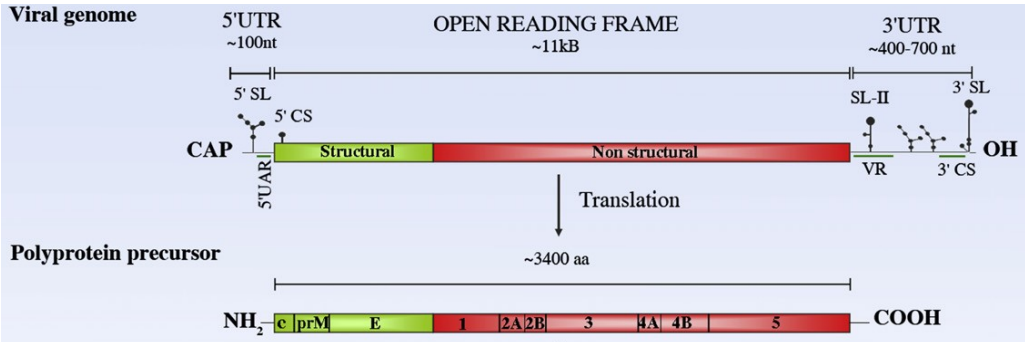


Fig. 2 Genome of Flaviviruses
The whole genome consists of a single, positive stranded RNA and contains only one open reading frame. The genome is flanked by short non-coding regions – the 5' and 3' UTR. Furthermore, the genome is capped at its 5' end, whereas the 3'end does not contain a polyadenyl tract. The genome is translated as one polyprotein [3].

1.4 Viral proteins

The polyprotein of YFV consists of 3411 amino acids [1]. As can be seen in Fig. 3, the N-terminal of the polyprotein codes for the three structural proteins: the RNA associated C protein, the pre-M protein and the E protein. The C-terminal of the polyprotein codes for seven non-structural proteins, namely NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 [Fig. 3]. The

functions of some of these proteins are still not fully understood, although it is assumed that these proteins play a major role in the replication of viral RNA. The structural proteins are responsible for virion binding to host receptors, virion stabilization and capsid formation, whereas the non-structural proteins are suggested to be involved in viral replication processes and pathogenesis [23].

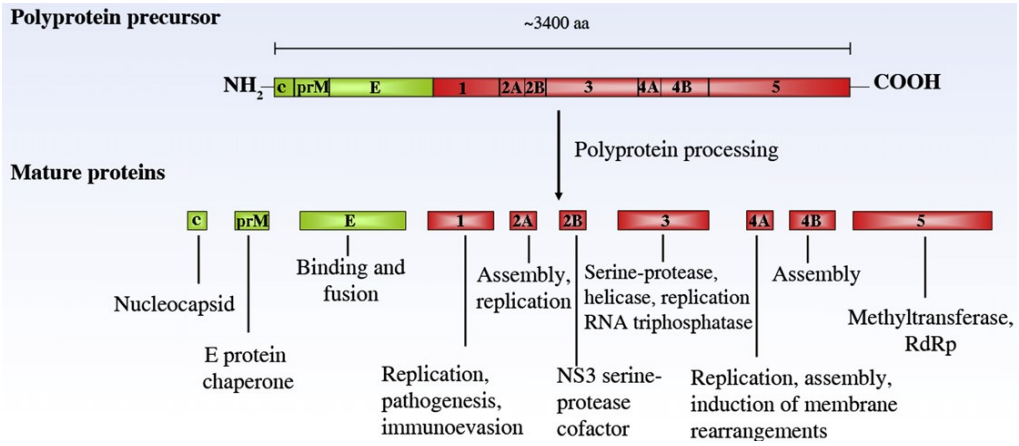


Fig. 3 Polyprotein processing
The polyprotein precursor codes for the three structural proteins, C protein, PrM protein and E protein and the non-structural proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 [3].

1.4.1 Flavivirus Structural Proteins

The glycosylated E protein (gE) is the major surface protein forming spike-like structures, which are projected out of the membrane. The main task of this protein is to mediate binding of virions to target surfaces and fusion with the membrane. Three structural domains have been identified for the E protein of dengue virus and tick–borne encephalitis virus. The first domain forms a ß-barrel structure, the second domain is the dimerization domain and the third domain is an immunoglobulin-like domain. It is assumed that this domain is responsible for receptor binding. Furthermore, it shows most of the antigen epitopes, which determine the strain and the type of the virus [24, 25]. In recent studies, a binding pocket between domain I and domain II could be identified. The binding of the ligand leads to conformational changes, which are necessary to trigger membrane fusion [26]. As it is assumed that Flavivirus structural proteins are similar, the same findings could apply for YFV E protein [11].

The pre-M protein is the precursor protein, which is the glycosylated membrane protein. During the passage through the Golgi apparatus the aminoterminal parts of the pre-M protein get

cleaved by the cellular protease furin into the non-glycosylated M protein. The cleavage of the precursor to the actual functioning protein is very important for the infectivity of the virus particles as this induces the fusogenic properties of the E protein, meaning the fusion of the virus membrane with endosomes after adsorption of the virus. The M protein chaperones the E protein through the secretory pathway and is responsible for the stabilization of this protein [11].

The C protein, which is responsible for the formation of the capsid, embraces the viral genome. It contains many alkaline amino acids, which individually interact with the viral genome inside the capsid. Furthermore, a C-terminal hydrophobic anchor in nascent C protein has been recognized to serve as a signal peptide for ER translocation of prM. This anchor is then cleaved from the mature C protein by the viral serine protease to generate the mature form of the C protein [1]. The C protein is reported to fold to a dimer, with each monomer containing four α helices. In recent studies, positively charged and hydrophobic residues could be identified, suggesting two separate surfaces responsible for RNA binding and membrane interaction [27].

One of the major gaps in understanding viral RNA synthesis is the question of the exact mechanisms of C Protein association with the newly synthesized viral RNA. Furthermore, it has been suggested that 5' and 3' complementary sequences, found at each end of the genome, associate through base pairing, thereby forming a circular conformation of the viral RNA. Studies have shown that genome cyclization is significant for viral replication. It would be interesting, to see with the help of life cell imaging microscopy, what role the C protein plays in genome cyclization, as the 5' complementary sequence was found to lie within the coding region of the C protein. In addition, a clearer understanding of viral RNA association with the C protein could be gained [28].

1.4.2 Flavivirus Non-Structural Proteins

As already mentioned before, it is assumed that the non-structural proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 encoded by the C-terminus of the polyprotein play a major role in RNA replication [28].

NS5 fulfils two functions – for one it is the RNA dependent RNA polymerase and second it is thought to be the methyltransferase, which is responsible for the formation of the RNA cap [29, 30].

The NS3 protein contains catalytic residues at its N-terminal, which are constituent parts of the viral serine protease. For the activation of this protease the NS2B protein is required as cofactor

[31-33]. The other parts of NS3 code for an RNA triphosphatase, which is proposed to play a role in RNA capping [33] and for an RNA helicase, which shows nucleoside activity [32, 34]. To date the functions of NS2A, NS4A and NS4B are still unclear. It is known that these proteins are small and hydrophobic. Studies have shown that they are involved in anchoring the viral replicase to cellular membranes [35].

The remaining NS protein, NS1, is a glycoprotein, which is highly conserved in the flavivirus genus, existing in intracellular, cell surface and secreted forms [36, 37]. The uniqueness of the protein is that it is exposed to the surface by virus-infected cells as well as secreted by infected cells. Furthermore this protein contains 12 invariant cysteine residues and two potential N- linked glycosylation sites [29]. It is inserted into the endoplasmatic reticulum through a signal sequence, which is located in the C terminus of the E protein and processed by host signal peptidase [38]. The NS1 protein is produced through cleavage from NS2A by an unknown endoplasmatic reticulum- resident host proteinase, dimerizing shortly after synthesis. Although it is known that it interacts with cellular membranes, it does not contain membrane – spanning segments. Therefore, the mechanism for the association with cellular membranes is still unclear [39].

Interestingly, it is proposed in several studies that NS1 plays an important role in the immune response of the host cell. It could be shown in experiments that animals vaccinated with the subunit of the NS1 protein or the NS1 protein produced by viral vectors, were protected against Flavivirus infections [40-44]. Furthermore West Nile NS1 is able to inhibit the formation of the membrane attack complex (MAC – complex) in the alternative complement pathway, hence evading the immunological defense mechanism [45, 46].

1.5 Flavivirus life cycle

Upon virus attachment, which is mediated through the envelope glycoprotein to host receptors, the virus gets endocytosed into vesicles, the so-called endosomes. Acidification of these endosomes leads to conformational changes in the E protein. This change in conformation induces the fusion of virus and host membranes, so that the viral genome gets released into the cytosol [24, 47]. In the cytosol, the nucleocapsid is first uncoated. This is followed by the translation of the viral RNA into a polyprotein, which then gets cleaved into the structural and non-structural proteins. The viral RNA also serves as template for the replication [23].

In the cytosol, the viral RNA binds to cellular ribosomes with the cap structure of the 5'end. During translation, the translation complex is translocated to the endoplasmatic reticulum (ER).

This process is initiated by the hydrophobic domain of nascent C protein, which serves as signal peptide. The interaction of this signal peptide and cellular signal recognition particles mediate the transport of the nascent polyprotein to the ER membrane. Co-translational procession of the polyprotein by a combination of cellular and viral proteases leads to the production of the mature structural and non-structural proteins (NS). One of the products cleaved - NS5- is a RNA-dependent RNA polymerase, responsible for the synthesis of a (-) sense RNA molecule, which acts as the template for the synthesis of the genomic progeny RNA [23].

The virus replication and assembly both take place in the cytoplasm on the surface of the endoplasmatic reticulum. The reason for this could lie in the surface structure of this organelle, which is organized in layers. These layers act as scaffolds and form vesicle packets, which help anchoring viral RNA, viral proteins, and possibly host cell factors, so that they can form the viral replication complexes [48]. Within these vesicle packets, the encapsidation of the genome RNA and the association of this with the viral glycoproteins take place. For the encapsidation, positive charges distributed throughout the C protein interact with the negatively charged phosphate backbone of the RNA. To date, the exact mechanisms of encapsidation and how the C protein recognizes the viral RNA are still not fully understood. However, some structural proteins, NS2A und NS3, could be identified responsible for genome packaging and nucleocapsid assembly [49]. Budding of the association of C protein with RNA genome and the viral glycoproteins into the ER lumen gives rise to the immature particle. The immature virion is transported to the Golgi apparatus in the ER-derived lipid bilayer, which contains heterodimers of the PrM and E proteins [50]. In here post-translational modification of the viral glycoproteins takes place. PrM inhibits premature fusion of the virus with the membrane of the transporting vesicle, during its transport to the Golgi apparatus. The furin-mediated cleavage of PrM to M triggers virus maturation in the trans-Golgi network, from where they are transported to the cell surface and through exocytosis or lysis of the cell released [49, 51, 52].

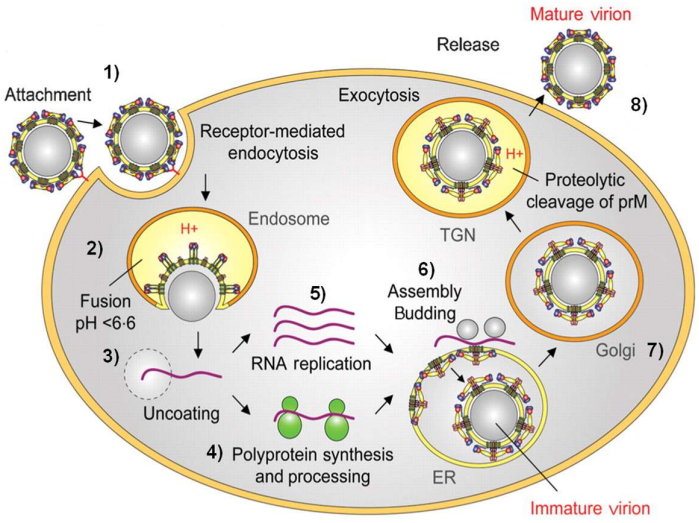


Fig. 4 Virus life cycle
The life cycle of Flaviviruses consists of eight different steps. 1) Virions bind to specific receptors, which induce endocytosis of the virus in so called endosomes. 2) Acidification of the endosomes triggers a conformational change in the E protein, which leads to the dissociation of the viral capsid in the cytosol, 3) where viral RNA is uncoated. 4) The 5'cap of the genome binds to the ribososmes of the endoplasmatic reticulum and triggers the translation of the genome into one polyprotein using the host cells own translation machinery. Nascent polyprotein is translocated co-translationally with the help of the signal peptide of nascent C protein to the ER membrane. This polyprotein is then cleaved into the final functioning proteins by viral and cellular proteases. 5) The NS5 protein, the RNA-dependent RNA polymerase, synthesizes a (-) sense-RNA, which serves as template for the genomic progeny RNA. 6) Virus assembly of viral RNA with C, PrM and E proteins take place on the surface of the endoplasmatic reticulum. This association buds into the ER lumen to form the immature virions. The assembled, immature virions are then transported in an ER-derived lipid bilayer to the Golgi apparatus. 7) In here virus maturation takes place by the cleavage of the PrM to M. 8) In the last step virions are released out of the cell through exocytosis or lysis of the cells [6].

1.6 Ecology and Epidemiology

YFV is maintained in nature by alternate infection of vertebrate and invertebrate hosts. Replication and amplification can occur in both hosts. Infections of humans occur accidently, when they intrude into the natural ecosystem of the other hosts. Human infections are not essential for the life cycle of the virus. However, for YFV, humans can be the major vertebrate host in an urban ecosystem and then the virus is maintained by alternate transmission from humans to mosquitoes [43].

There are three different transmission cycles characterized for the YFV: Jungle cycle, the Savannah cycle and the urban cycle. All the three transmission cycles for yellow fever in Africa and South America are depicted in Fig. 5.

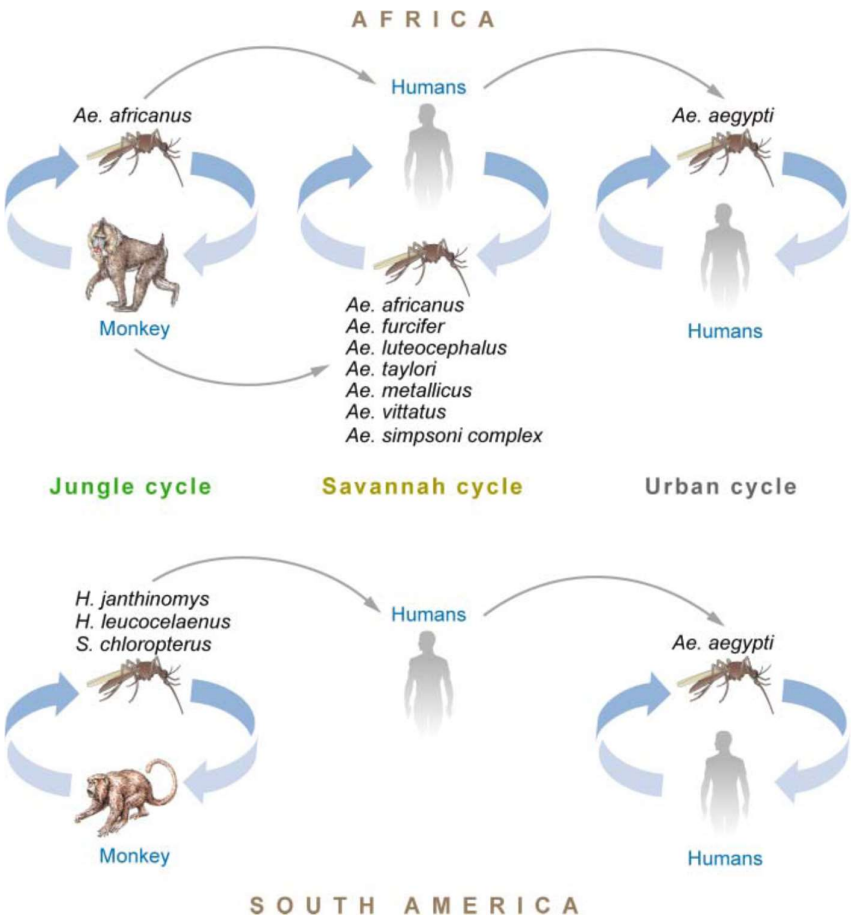


Fig. 5 The three transmission cycles for YFV in Africa and South America, showing the various vectors involved.
In the jungle cycle, also called enzootic cycle, monkeys are the major vertebrate hosts, although viremia is short lived in them. Amplification of the virus occurs mainly in mosquitoes, which remain infected for life. Occasionally human infections might occur, if they venture into the forest. In the savannah cycle, forest mosquitoes invading the areas surrounding rain forests cause infections in humans, which are then transmitted to other humans. The urban cycle involves the transmission of the infection from human to human by the *Aedes aegypti* mosquito [5].

1. **In the jungle cycle** or the enzootic cycle, monkeys are the major vertebrate hosts. However, the viremia is short lived in the monkeys and therefore they are only the transient host. The amplification of the virus mainly occurs in the mosquitoes which remain infected for life and are also able to transmit the infection transovarially. Occasionally human infections might occur, if they venture into the forest. The enzootic cycle has been recognized in South America and parts of Africa [44].

2. **The Savannah Cycle** is the most significant epidemiological form of yellow fever regarding human infection. Forest mosquitoes invading the areas surrounding rain forests cause yellow fever infections in humans. Thereafter, human to human transmission maintains the epidemic in widespread proportions [45].
3. **The urban cycle** involves the transmission of the infection from human to human by the *Aedes aegypti* mosquito. The virus is able to infect and amplify in the mosquito to a high titer viremia and can be transmitted through the saliva. Extensive mosquito control could eradicate yellow fever in many South American towns, but the disease could resurface due to the reinfestation with *Aedes aegypti* [45].

YF is prominent in tropical regions of Africa and South America, where the virus is persistently present at low levels of infection [Fig. 6]. The viral presence amplifies frequently into regular epidemics leading to numerous deaths. There are 200,000 estimated cases of yellow fever (with 30,000 deaths) per year in these regions [13]. The exact factors involved and responsible for the cyclic appearance and disappearance of virus activity are still not known and remain of immense interest. Furthermore, it still is not fully understood how the virus can survive between epidemics. In several studies, it was proposed that the virus survives dry seasons through vertical transmission from infected female mosquitoes to their eggs. Virus particles are stable for long periods. Thus, once climate conditions improve and the progeny emerge, virus particles can be reactivated [53, 54].

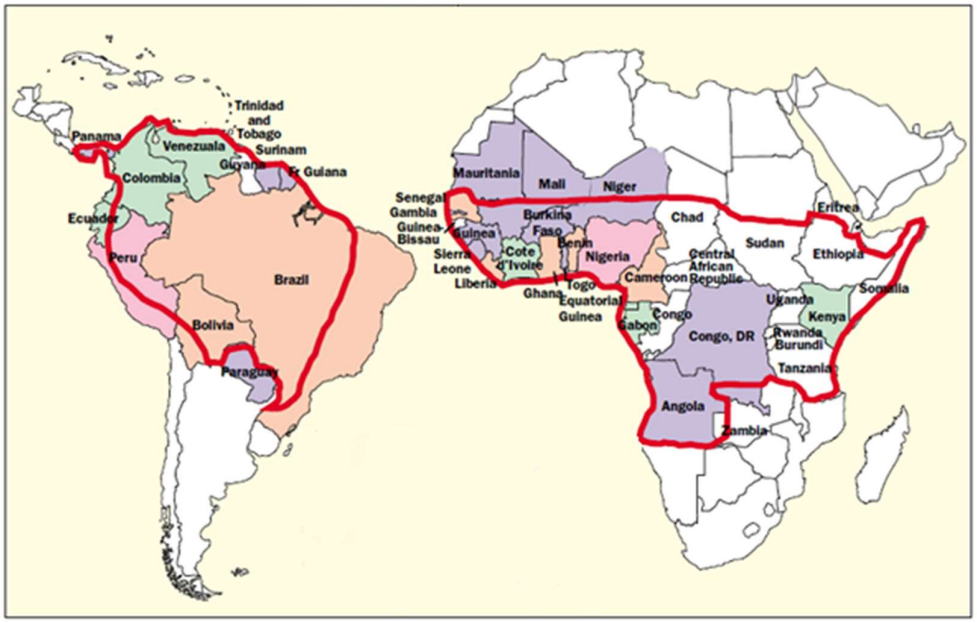


Fig. 6 Yellow Fever endemic regions, marked by red boundary [2].

Interestingly, yellow fever has not been reported in Asia despite the presence of a large susceptible human population and the vector *Aedes aegypti*. It is suggested that this coincidence is linked with cross-protection provided by hyperendemic dengue and also because of the low competence of the prevalent populations of asian *Aedes aegypti.* Furthermore, any occurrence of yellow fever in a remote area could be contained in absence of widespread travel to the region [55].

1.7 Clinical features and Immune response

The inoculation process of the YFV commences when an infected female mosquito feeds on the blood of the host. During this process 1,000 to 100,000 virus particles are transferred intradermally. Replication of the virus takes place in the local dendritic cells in the epidermis immediately after YFV infection and the lymph nodes are the first organ reached by the virus. This enables virus particles to spread to other organs like liver, kidney, heart and thymus leading to damage of organs, which is caused by the host immune response to the virus or cytopathic effect of the virus. Unfortunately, the full pathogenesis of yellow fever virus is still not fully understood. Therefore, questions regarding the role of the immune response to virus infection and especially the role of the cellular immune response have yet to be appointed [56].

Once the YFV is transmitted to a human body, the incubation period can extend from three to six days. Though some infected individuals may not present any symptoms at all, others exhibit two distinguishable disease phases - the acute phase and the toxic phase. In the acute phase, the period of infection, symptoms like fever, muscle pain, headache, shivers, loss of appetite and nausea occur. This phase corresponds to the phase of viremia, as infectious virus particles can be detected in the blood (up to 10^5-10^6 infectious particles per mL) [57].

In most patients, the fever subsides after three to six days. This phase is called “period of remission” and can last up to 48h. In this phase, the host’s antibodies and the cellular immune response clear the virus. Approximately 75-85% of people affected, recover at this stage and all the symptoms disappear slowly. However, in around 15% of people affected the toxic phase occurs within 24 hours. In this phase, the fever reappears accompanied by the dysfunction of several organ systems. There is a rapid onset of jaundice through the destruction of hepatocytes and that is why the disease is known as yellow fever. Furthermore, bleeding from the mouth, nose, eyes and stomach can occur and can cause appearance of blood in the vomit (Black

vomit). There is significant organ damage followed by death in around 50% of the patients in the toxic phase [4].

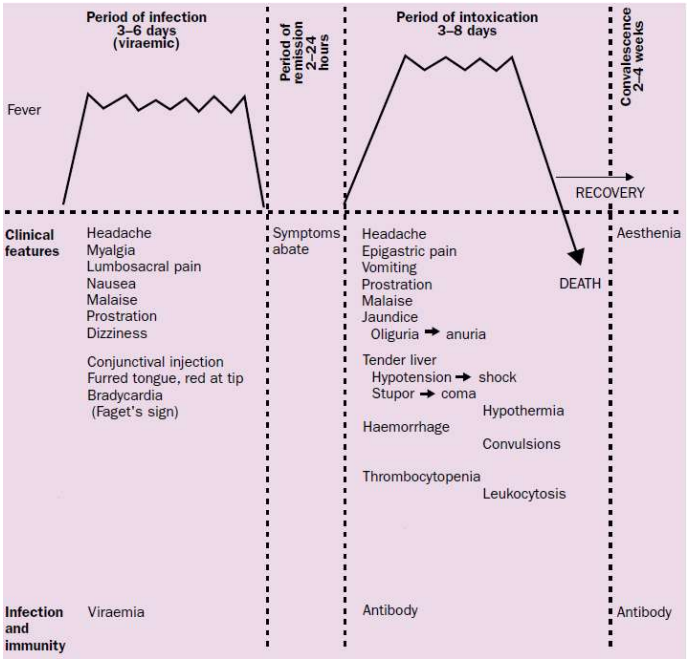


Fig. 7 The three different phases of yellow fever infection
The illustration shows all major clinical features of this disease. The first phase is marked by symptoms like fever, muscle pain, headache, shivers, loss of appetite and nausea. These symptoms disappear in the "period of remission". In about 15% of the cases this phase is followed by the last stage – the period of intoxication or toxic phase. In this phase, most of the symptoms appear again, only with much more intensity leading to organ damage, which is often followed by death. In the first phase, viraemia is quite high. In the intermediate phase remaining virus in the blood is cleared, leaving non-infectious immune complexes [4].

The body reacts to the YFV infection by a rapid specific immune response. Although the mechanisms of the immune response elicited after YFV vaccination are still not fully understood, the humoral response has been characterized. The titer of IgM antibodies increases slowly in the first week, peeks in the second week and then declines over several months. Neutralizing antibodies are present from the end of the first week after the onset of the disease and persist for many years. These antibodies are capable of keeping infectious agents from infecting cells by inhibiting their biological effect, for example by generally blocking the receptors on the cell or the virus [58].

Interestingly, patients who have been previously infected with certain heterologous Flaviviruses are partly cross-protected against YFV. Even so, this cross-protection is only provided by infection with some African Flaviviruses, like Zika and Wesselsbron, and in particular with dengue [4].

1.8 Diagnosis

One of the difficulties in the diagnosis of YF is the similarity of the disease pattern to other diseases caused by pathogens. Leptospirosis and louse-borne relapsing fever (*Borrelia recurrentis*) for example are also characterized by jaundice, hemorrhage, disseminated intravascular coagulation, and a high case fatality rate. Viral hepatitis, severe malaria (Blackwater fever), dengue and Crimean-Congo hemorrhage also exhibit clinical features which are similar to YF and hence have to be differentiated [59].

Since YF is difficult to be diagnosed based on the symptoms, specialized laboratory tests and expert opinion are required to confirm the infection. The tests are typically based on the detection of viral RNA in the blood during the initial phase of infection and the detection of viral antibodies in the patient's sera [58].

Liver samples from patients with fatal disease are required for histopathologic studies. However, biopsies are contraindicated in living patients due to the risk of hemorrhage. In the case of an infection with YFV, formation of Councilmen bodies, typical midzonal necrosis and microvascular steatosis can be seen in the obtained liver samples. It is advised to be cautious in the interpretation of the results as some other diseases like Lassa fever, Ebola, Marburg, Viral hepatitis Crimean-Congo, hemorrhage and Leptospirosis exhibit the same histopathologic features [59].

Virus isolation from the blood is difficult and can only be done in the period of infection during the first three to six days after the onset of fever. In this period, virus titers in the blood can be up to 10^5-10^6 infectious particles/mL. To isolate the virus from an infected person inoculation of for example the serum sample can be done in the cerebral system of suckling mice, thorax of mosquitoes or in cell cultures. In viral diagnosis, viral detection can be confirmed by PCR (Polymerase Chain Reaction) to detect the viral genome [60, 61]. Molecular techniques for virus diagnosis are applied more frequently than ever before, especially for clinical samples that were negative by virus isolation, as they are fast and sensitive. Hence, they are used as standard methods. Especially Real-Time PCR, which not only allows the detection of viral nucleic acid but also its quantification, is the method of choice. However, disadvantages of PCR-based diagnosis techniques are that they can be only applied during the acute phase of the disease, when the patient remains viremic. Moreover, the diagnostic samples have to be stored properly at -80°C, as otherwise the virus viability and RNA is degraded [60].

Immunological techniques are used for the detection of viral antigens or specific antibodies in serological tests. These assays are useful after the end of the first week of symptomatic disease when the immune response becomes detectable. Hence, these tests are mostly used for the diagnosis of YFV. Serological tests include ELISA, hemagglutination inhibition, neutralization assays and complement binding assays. These methods rely on the measurement of the concentration of specific antibodies or antigens in the serum. It is possible to detect IgM- and IgG- antibodies in the serum around eight to fourteen days after the YFV infection. After six to twelve months the IgM antibodies disappear again. Neutralizing IgG antibodies persist throughout the lifetime and protect the host from a reinfection [4].

Complications in serological diagnosis may occur in individuals who already have been infected with other Flaviviruses, hence causing cross-reactions. This is likely to happen in regions where multiple Flaviviruses are endemic [61].

For the conformation of an YFV infection the neutralization assay is considered the most specific technique and hence, is the most commonly used assay to date. According to WHO guidelines this assay is mandatory to confirm a YFV infection. Furthermore, the neutralization assay is used to confirm the immune response elicited after YFV vaccination. Vaccinated people are considered protected when they have a titer above 1:10 [13]. However, the disadvantage of this technique is that it takes time to get the result so that it cannot be used for early diagnosis. Also, the performance of the assay and the interpretation of the results can only be carried out by highly trained laboratory staff in special laboratories with cell culture and bio containment facilities.

1.9 Treatment and Prevention

To date there is no specific antiviral treatment for yellow fever. Several studies have shown that ribavirin is ineffective in patients suffering from a YFV infection. Furthermore, passive antibodies and interferons are only of help if administered before or within a few hours of infection. This means that patients can merely receive supportive treatment for symptomatic relief rather than combating the cause of the infection. However, this treatment does not provide any help to patients who are already diagnosed to be in the toxic phase of a YFV infection. This underlying fact together with the increasing number of YFV cases makes it a subject of utmost importance in terms of disease control and prevention measures. The use of pesticides and the eradication of mosquito-breeding sites are some of the control measures that are currently applied. However, the use of large scale insecticides to eliminate enzootic circulation can be

harmful for the environment. Besides, the vaccination of wild animals with the YF-17D is nearly impossible, as they are spread out and mostly situated in remote areas. Nevertheless, one promising control measure for the reduction of urban mosquito transmission was introduced by Van den Hurk et al. They have developed a very interesting strategy, where mosquitoes are infected with intracellular *Wolbachia spp.* bacteria. These bacteria have shown to suppress the replication of some arboviruses including dengue and YFV [62].

Till date the most effective way in reducing the disease spreading, is still by vaccination with the YF-17D vaccine. However, more and more reports appear where vaccination campaigns have to be delayed due to the shortage of the YF vaccine. In 2010, only one vaccination campaign could be carried out. Currently 75 million doses of the yellow fever vaccine are being produced every year; whereas the demand for the vaccine was already over 105 million doses back in 2010. The demand for the vaccine will continuously increase due to preventive campaigns that are implemented in Africa. Additionally, countries at lower risk have also started to introduce preventive immunization campaigns and routine childhood immunization against YFV. This therefore constantly increases the worldwide demand of the vaccine. The risk of large outbreaks is further enhanced through the presence of non-immune populations resulting from migration movements from rural to urban areas in Africa with high vector densities. Genuine commitment, both in national as well as global level, is required in order to maintain a secure supply of the vaccine to prevent major epidemic outbreaks.

1.10 The Yellow Fever Vaccine History

In the 1920s, great effort has been taken in isolating wild-type YFV. In 1927, isolation of two wild-type virus strains – one from Ghana, called Asibi strain and the other one from Senegal, called French viscerotropic virus, was successful for the first time. These two virus strains were serially passaged either in mouse brain to develop an attenuated virus variant named French neurotropic vaccine (FNV), or in chicken tissue to yield the attenuated subculture, called 17D. Both vaccines induced a protective immune response in humans and primates. 17D has several mutations in the structural and non-structural genes of the virus, which also resulted in a loss of the ability to infect vectors. Consequently, the vaccinated individuals cannot serve as a source of infection for mosquitoes. However, little is known about the mutation and thus mechanisms responsible for this attenuation [63, 64].

Unfortunately, FNV caused severe post vaccine encephalitis in children with a case fatality rate of 40%. Therefore, FNV production was stopped in 1982. Since then, the 17D strain has been

further passaged in chicken tissue, which resulted in two sub-strains, namely 17D-204 and 17DD [65]. Both of these substrains are used as vaccines, with which more than 400 million people have been vaccinated. YF 17D vaccine is considered as one of the most effective and safe vaccines in use today with favorable cost-benefit ratio in endemic countries [66]. The WHO has recommended revaccination with the 17D vaccine every 10 years for people living in endemic regions of Africa. However, in March 2013 the Strategic Advisory Group of Experts (SAGE) assembled by the WHO held a meeting where this recommendation was revised. Their investigations revealed only 12 cases of yellow fever infections in vaccinated persons within five years of vaccination. Given the fact that more than 600 million doses of the YF vaccine have been administered, the number of re-infections is quite insignificant. Therefore, SAGE concluded that a single dose of YF vaccine is sufficient to maintain life-long immunity and that vaccine failure is extremely rare.

Interestingly, yellow fever 17D vaccine virus can be employed as vector for foreign genes by exchanging the E protein of YFV-17D with corresponding genes of other Flaviviruses. This recombinant yellow fever vaccine virus establishes the basis for the development of recombinant, live vaccines against other Flaviviruses, like dengue or West Nile. Furthermore, recent studies have shown that the use of this recombinant yellow fever vaccine virus induced a cytotoxic cell response to an expressed tumor antigen [67, 68].

Rare cases of adverse events have been described in yellow fever vaccinated persons [156-160]. Therefore, the development of an alternative inactivated YF vaccine has been in the focus of a lot of research groups. This would be especially helpful for people at higher risk of adverse events from the live vaccine. Additionally, this would lower the risk-benefit threshold and thus assuring high levels of yellow fever vaccine coverage [69]. In 2010 the research group of Monath et al [70] succeeded for the first time in developing an inactivated whole virion vaccine based on the 17D virus. This virus was inactivated with ß-propiolactone and adsorbed to aluminum hydroxide [71]. Although this inactivated virus looked promising initially the question would be if one single dose could provide life-long immunity as compared to the YF 17D vaccine.

1.11 Motivation and Objectives

Flaviviruses are still the cause of extensive infectious disease morbidity and mortality across every continent [12]. Twenty-two out of twenty-seven mosquito-borne Flaviviruses, twelve out of thirteen tickborne Flaviviruses and five out of fourteen Flaviviruses with no known vector

are responsible for causing diseases in humans. Important human pathogens of this genus are yellow fever virus, dengue, West Nile, Japanese encephalitis and tick borne encephalitis virus [1]. YFV is one of the RNA viruses which has been investigated thoroughly over the last century. It was detected as the first human pathogenic virus which led to the comprehensible interest in its research. Additional significant interest came through the development of the 17D vaccine [12]. Although being an attenuated live vaccine strain, it can still induce a protective immune response in humans, making it an easy tool for molecular biological experiments [72].

Over the past two decades the number of yellow fever cases has increased dramatically. The reasons for this are deforestation, urbanization, population movements, climate change and declining population immunity to the infection. Therefore, major concerns of public healthcare institutions are to maintain high levels of immunity in the population of endemic regions which can be carried out by implementation of specific preventative health measures for curtailing outbreaks and thereby reducing disease burden.

The maintenance of high levels of immunity in the population is achieved by routine childhood immunization. In 1991 YF vaccination has been incorporated in the Expanded Program of Immunization (EPI) in Africa [73]. In order to avoid the loss of opportunities for vaccination and due to better cost/benefit ratio, simultaneous administration of different vaccines is recommended. According to the EPI, children at 9 months of age have to be vaccinated with YF 17D and measles vaccine concomitantly. However, only limited data is available on the safety and immunogenicity when YF 17D vaccine is administered simultaneously with other vaccines. In a previous study, it could be shown that simultaneous administration of YF 17D and measles vaccines had no effect on the safety or immunogenicity of both the vaccines in children [74, 75]. Nevertheless, one study indicated that the administration of YF 17D and measles, mumps and rubella (MMR) vaccines can lower immunogenicity significantly in children [76]. Therefore, it is crucial to conduct more clinical studies, where simultaneous administration of vaccines is closely analyzed. Besides, each vaccine has to be tested along with the new vaccine in order to detect any kind of interference before introducing these in the EPI. In this context, one of the objectives of this PhD was to analyze the immunogenicity of the YF-17D vaccine when administered concomitantly with measles vaccine and a newly developed monovalent, serogroup A meningococcal conjugate vaccine (PsA-TT, MenAfriVac). MenAfriVac was developed through the program of the Meningitis Vaccine Project (MVP), which is a partnership between WHO and PATH [77-81]. Moreover, results on seroconversion rates are presented as to date there is still only limited data available on the efficacy of the YF 17D vaccine in infants.

Early implementation of specific preventative health measures for curtailing outbreaks and reducing disease burden are achieved by the development of so called epidemiological surveillance programs, which are focused on rapid, accurate laboratory diagnosis [4]. However, the clinical diagnosis of YFV is difficult. The reasons are case-by-case differences in severity and also differences in the observed symptoms, which are similar to symptoms caused by dengue fever, viral hepatitis, Leptospirosis, Lassa fever and Ebola fever, just to name a few. Thus, the diagnosis has to be confirmed by laboratory tests, which are based on molecular and serological methods for the detection of YFV genomic sequences, virus encoded antigens or virus-induced antibodies. Although molecular techniques, such as the polymerase chain reaction, can be more sensitive and specific than serological methods, these can only be applied in the acute phase, while the patient remains viremic. Therefore the disease is mostly confirmed by serological tests (serodiagnosis) [4]. Nevertheless, the existence of cross-reactive antibodies produced in response to other simultaneous and/or previous Flavivirus infections poses a serious problem for the differential diagnosis. This is especially the case for endemic regions, where many flaviviral infections are circulating and hence a correct diagnosis is required. However, serological tests as the neutralization assays are considered as one of the most specific tests currently available in serological methods [82]. One of the disadvantages is that these assays can only be performed by highly trained laboratory staff and only in special laboratories with cell culture and bio-containment facilities [4].

As the laboratory of AG Niedrig was a diagnostic laboratory, one of the aims of the group was the continual improvement of already standardized assays for detecting yellow fever and dengue virus amongst others. Although these standardized methods achieve good and reliable results, improvement is sought in specificity, saving time, expenses and lowering the volume of samples for the tests. Hence, the second project was to establish a new specific indirect ELISA for the detection of YF IgG and IgM antibodies in serum samples. In order to establish such an assay, serum samples of the immunogenicity studies, which had a known *NT*, were used. The results were then compared with the *NT* obtained by the already standardized microneutralization assay. Furthermore, the third project dealt with the establishment of common virological techniques for the analysis of YF on a novel tool, namely the xCELLigence™ system. This system is based on cell impedance measurements in real time. The results obtained by this system were compared to the established microneutralization assay in order to see if this system could serve as an alternative to classical virological methods.

2 Methods

This chapter deals with all the methods, which were used during the time of the PhD work. It is divided into seven sub-chapters. These are cell culture, molecular biological, microbiological, protein biochemical, immunological and virological methods. In these chapters, all the protocols according to topic area are illustrated. The chapter ends with presenting a novel tool - the xCelligence™ System and introduces the protocols, which have been established on this new system.

2.1 Cell culture

As cell lines Vero E6 cells and Pig kidney epithelial cells (PS cells) were used. Both these cell lines derive from kidney epithelial cells. However, Vero E6 cells are extracted from an African green monkey whereas PS cells originate from a pig. These cells are used as they are susceptible to a wide range of Flaviviruses.

2.1.1 Cultivation of adherent cells

All cells were cultured in 75 cm^2 flasks at 37°C, with 5% CO_2 in 25 mL Dulbecco´s Modified Eagles Medium (DMEM). The medium was supplemented with 10% FCS and 1% L-Glutamine.

When the cells reached a density of 90% - 100% the old medium was removed and the cells were washed with PBS. As Vero E6 cells as well as PS cells are adherent they needed to be detached from the culture dish first. This was done with the help of HyQtase™, a trypsin-free blend of proteases and collagenases, which degrade surface proteins of the cells. After washing the cells, 5 mL of HyQtase™ was added and the cells were incubated for 10 min at 37°C. When all cells were dissociated from the culture flask, another 5-10mL of DMEM supplemented with 10% FCS was added to stop the reaction of HyQTase™ and the cells were centrifuged for 5 min at 1100 x g. The supernatant was decanted and the cells were gently re-suspended in 10 mL of fresh DMEM supplemented with 10% FCS by pipetting up and down to singularize cell colonies. In order to re-seed cells in lower densities one part of the cells was transferred to a new 75 cm^2 culture vessel and filled up with fresh medium. The number of transferred cells was determined by the growth rate. Typically, cells are split from 1:2 to 1:10 depending on the cell lines.

2.1.2 Cell counting

The cell concentration per mL was determined with the help of the Neubauer cell counting chamber. For this 10 µL of the cell suspension was put into a Neubauer counting chamber and all 4 x 16 squares were counted in the chamber. The cell number n_c per mL was calculated according the formula $n_c = n_{cc} / 4 \times 10^4$, where n_{cc} is the counted cell number. If the cell suspension is diluted, the dilution factor has to be taken into account.

2.1.3 Preservation and storage of the culture cells

In order to maintain backups of the cells without the associated effort of feeding and caring for them the cultured cells were preserved. For this the re-suspended cell suspension was collected in a centrifuge tube and spun down for 10 min at 1100 x g in a cold centrifuge (4°C). The supernatant from the centrifuged cells was removed and the cell pellet was re-suspended in a cryoprotective medium containing 10% DMSO and 90% FCS. Dimethylsulfoxide (DMSO) is a cryoprotective agent, which lowers the freezing point as freezing can be lethal to the cells due to the effect of damage by crystalline structures. 1mL of the DMSO containing cell suspension was added to each stocking vial and put into the -80°C refrigerator. After 1-2 weeks, the cells were transferred to the liquid nitrogen freezer for permanent storage.

2.2 Molecular biological methods

2.2.1 DNA sequencing

One of the most powerful techniques in molecular biology is the DNA sequencing i.e. the determination of nucleotide sequences of specific DNA fragments. There are many ways to determine DNA sequences but one of the most commonly used methods for the determination of nucleotide sequences is the dideoxy sequencing method, also known as Sanger method or chain terminator method. The basic principle of this method relies on the extension of a known DNA fragment in a conventional polymerase chain reaction. For this reaction special nucleotides, besides primers and the DNA polymerase, are required. These nucleotides, which are called 2',3'dideoxynucleotides and are not found in DNA, lack the 3'hydroxyl group, because of which they cannot form phosphodiester bonds. Phosphodiester bonds are required for the incorporation of new nucleotides into the newly synthesized DNA strand. Hence, DNA chain elongation is terminated as soon as a dideoxynucleotide is inserted. The insertion takes place randomly resulting in DNA fragments of varying length. In order to distinguish between

the different nucleotides, the sequencing reactions are carried out in four different tubes. Each tube contains the same DNA template, all four standard deoxynucleotides, DNA polymerase and only one kind of dideoxynucleotide. The separation of the newly synthesized and labelled DNA is accomplished by polyacrylamide gel electrophoresis, where each reaction is run in one of four adjacent lanes (A, T, G, C). The DNA bands are then visualized either by autoradiography or by UV light, so that the DNA sequence can be read off directly from the pattern of alternating bands in the lanes corresponding to the terminal base of the fragment [83].

For the sequencing of plasmid DNA, the BigDye® Terminator v 3.1 Cycle Sequencing Kit was used, which allows automated sequencing. This kit uses different dyes for each dideoxynucleotide, so that the reaction can be carried out in one single tube.

After synthesis, the DNA fragments were separated by size with the help of capillary gel electrophoresis, which is a variation of the usual polyacrylamide gel electrophoresis. The major difference is that the gel matrix is embedded in the capillary itself, which has a diameter of 25-100 µm, and that the migration and separation, respectively, occur in this. Due to the high ratio of the surface area to the volume of the capillary, higher electric fields can be employed as heat, which is produced during electrophoresis, can be dissipated more easily, hence decreasing the run time and improving DNA resolution [83].

Furthermore, there is an argon ion laser attached to the capillary. When voltage is applied the DNA, fragments start to migrate through the capillary. During migration they pass the laser, which excites the fluorescein donor dye with which the DNA strands are labeled. As each of the four dyes emits light at different wavelengths the signals of all four dyes can be separated and detected in one scan. The resulting chromatogram can be converted into a sequence with the help of the software “Lasergene 8 SeqMan”.

2.2.2 Quantification of nucleic acid

The determination of the concentration of purified nucleic acid is achieved by spectrophotometric analysis as nitrogenous bases in nucleotides absorb ultraviolet light at a wavelength of 260 nm. At this wavelength, the sample is exposed to ultraviolet light and the the light that passes through the sample, i.e. the optical density (OD), is measured with the help of a photo detector.

For the measurement of the OD at 260 nm the sample was diluted in a total volume of 50 µL and the absorbance of it was measured in a micro cuvette. The concentration of the sample was calculated with the help of the following formula:

$$c[\mu g/mL] = OD_{260nm} x 50\,\mu g/mL\, x\, V$$

where V is the dilution factor and 50µg/mL is the multiplication factor used specifically for DNA.

The purity of the DNA preparation can be estimated by measuring the absorbance at 260 nm and 280 nm. Pure DNA preparations should have ratios between 1.8 - 2.0, lower ratios are an indication for protein contamination [83].

As an alternative, the NanoDrop™ ND1000, which is a full-spectrum (220-750 nm) spectrophotometer, was employed. This apparatus has the ability to measure very accurately1 µL of highly concentrated samples without dilution and without the use of cuvettes. The samples are directly placed on the so-called measurement pedestal of the apparatus and the samples can then be spectrally measured so that the concentration of nucleic acid in the sample can be determined.

2.2.3 Polymerase chain reaction

The polymerase chain reaction is used to selectively amplify specific DNA fragments with the help of two sequence-specific Primers and one thermally stable DNA Taq-Polymerase. A conventional PCR typically consists of 20-35 cycles, where each cycle comprises three temperature profiles, which are used to control the activity of the polymerase and the binding of primers. The first cycle begins by heating the reaction mixture to 94°C through which hydrogen bonds that hold the DNA strands together break, thus denaturing the DNA. After denaturing the DNA, the temperature is reduced to around 55°C so that the primers can form hydrogen bonds, or anneal to their complementary sequences in the target DNA. In the next phase, the temperature is raised to 72°C, which is the optimal working temperature for the Taq-Polymerase. In this phase polymerization of the primer takes place. The Taq-Polymerase attaches free Nucleotides to the 3'end of each Primer. After one complete cycle, there is one double stranded copy of the target DNA. The newly synthesized DNA fragment also serves as a template so that the number of newly synthesized DNA fragments grows exponentially after each cycle [83].

In the following table, the protocol of a typical PCR reagents mixture is presented. This protocol was used as a standard. In case of secondary structures 5% of DMSO was added to each reaction tube.

Table 1: Typical PCR reagents mixture

Reagents	Volume [µL]	Time [sec]	T [°C]	Cycle
dest. water	20.05	600	94	
10x reaction buffer	2.5	30	94	
dNTP mix (10 mM)	0.5	30	59	35
MgCl2 (50 mM)	0.75	90	72	
forward Primer (25µM)	0.5	300	72	
Reverse Primer (25µM)	0.5	Hold	4	
Taq-Polymerase	0.2			

2.2.3.1 Copy-DNA synthesis

For the quantification of the viral RNA load in a virus stock, the viral RNA had to be purified first and transcribed to cDNA by the reverse transcriptase. The purification was done with the help of the "QIAamp® Viral RNA Mini Kit" according to the manufacturer's protocol. For the synthesis of complementary DNA (cDNA) from RNA, SuperScript™II as reverse transcriptase and random hexamers, primers which bind the RNA randomLy, were used. Following reagents were mixed together for the PCR reaction:

Table 2: Copy-DNA synthesis reaction

Reagents	Volume [µL]
5 x Puffer I	2.00
DTT [0.1 mM]	2.00
dNTP-Mix [2.5 mM]	1.25
Random Hexamer [3µg/µL]	0.50
dest. Water	2.75
Mastermix	8.50
RNA	10.00
RNaseOut [40 U/µL]	1.00
SuperScript™ [200 U/µL]	0.50
Total volume	20.00

The purified RNA was heated up at 65°C for 10 min and afterwards 8.5µL Mastermix, 1µL RNaseOut and 0.5µL SuperScript™ were added. The mixture was shortly centrifuged and then put on ice for 5 minutes.

The PCR program was set according following scheme:

Table 3: Copy-DNA PCR program

Time [min]	T [C°]
60	42
5	93
Hold	4

2.2.3.2 SuperScript™ OneStep RT-PCR with Platinum®Taq

This kit was used to amplify a specific gene fragment with gene-specific primers out of viral RNA for cloning purposes. The special feature of this kit is that cDNA synthesis and PCR amplification occur in a single tube. This is achieved by the addition of SuperScript™ reverse transcriptase, gene-specific primers and Platinum®*Taq* Polymerase all in one single tube. The SuperScript™ reverse transcriptase uses gene specific primers to synthesise a specific cDNA fragment, which is subsequently amplified by the Platinum®*Taq* Polymerase. In the first step, the synthesis of cDNA, only SuperScript™ reverse transcriptase is active. ThePlatinum®*Taq* Polymerase is bound to an antibody in a complex, by which its activity is inhibited. After cDNA synthesis and before PCR amplification, the reaction is heated to 94°C, through which the antibody denatures from the complex and thus activates the Platinum®*Taq* Polymerase. Following protocol was used for the OneStep RT-PCR:

Table 4 OneStep RT-PCR reaction and the respective PCR program

Reagents	Volume [µL]	Time	T [°C]	Cycle
2x reaction buffer	12.50	30 min	45	
Primer sense (10µM)	1.00	2 min	94	
Primer antisense (10µM)	1.00	15 sec	94	
RT/Platinum *Taq* Mix	0.50	30 sec	55	40
DMSO	1.25	1 min/kb	72	
RNA	2.00	10 min	72	
ad *Aqua mol.*	25.00	Hold	4	

2.2.3.3 Colony PCR

A very simple, sensitive and specific method to screen for positive *E.coli* clones carrying the plasmid with correct incorporated DNA fragments is the colony PCR. For this method bacterial cells can be used directly, bypassing the time-consuming DNA isolation steps.

The PCR reagents mixture was prepared according the standard protocol (see 3.2.1) and 25µL was distributed to each PCR tube. A single colony was then taken with a sterile pipette tip and re-suspended in the PCR reaction tube. Afterwards the tip was put into 3 mL of LB containing ampicillin (80µg/mL), chloramphenicol (30µg/mL) and glucose (1%) and incubated over night at 37°C. The PCR was run according the temperature and cycler conditions presented under 3.2.1. Once this was completed, the entire reaction was loaded onto an agarose gel afterwards in order to analyze the amplified products.

After amplified products were analyzed, plasmids of the positive clones were isolated with the help of "Invisorb®Spin Plasmid Mini Kit Two" from Invitek according manufacturer's protocol [section 0]. The concentration of the isolated plasmids was measured by the NanoDrop™ ND1000 at 260 nm and sequenced subsequently for verification.

2.2.3.4 Mycoplasma PCR

In order to detect any contamination of the cell lines with mycoplasma, these are checked on a regular basis by PCR. This enables an amplification of a specific 280 bp-fragment of the 16S-rRNA gene, which can be detected afterwards by an agarose gel electrophoresis.

All the samples were first heated for 5 min at 95°C and then diluted at the ratio of 1:10. For the PCR the following mixture was prepared.

Table 5 Mycoplasma PCR reachtion and respective PCR program

Reagents	Volume [µL]	Time [sec]	T [°C]	Cycle
dest. water	15.15	600	94	
10x reaction buffer	2.5	30	94	
dNTP mix (2.5 mM)	0.5	30	59	35
MgCl2 (50 mM)	0.75	90	72	
GPO-3-Primer (50 µM)	0.5	300	72	
MGSO-Primer (50 µM)	0.5	Hold	4	
Taq-Polymerase	0.1			
	22.50			
Sample (1:10 diluted)	5			
Total	25			

2.2.4 Agarose gel electrophoresis

A very common and simple method used in biochemistry and molecular biology to separate DNA or RNA molecules by size is known as agarose gel electrophoresis. The basic principle of this method is the migration of negatively charged nucleic acid through an agarose matrix with an electric field. Molecules which are shorter move faster and further away than the larger ones. The DNA can be visualized in the gel by adding ethidium bromide. Ethidium bromide is a fluorescent dye, which binds to DNA by intercalating between the bases and glows when illuminated with ultraviolet light. The higher the agarose is concentrated, the smaller the pores in the agarose matrix. Usually agarose gels are made between 0.7% - 2%. For separation of about 5 kb big DNA fragments lower percentage (0.5%) agarose gels are made. For small fragments with approximately 500 bp 1% - 2% gels are made [83].

For the preparation of an 1% agarose gel, 0.5 g of agarose was mixed with 50 mL of 1x TBE in an Erlenmeyer flask and then heated in a microwave until the agarose was completely dissolved. Two point five µL of ethidium bromide was added and the mixture was cooled down at room temperature for approximately 5 min. After slowly pouring the gel into the tank and carefully inserting the comb, the gel was left for polymerization for about 20 min. After the gel had set, the comb was carefully taken out and 1x TBE running buffer was added until the gel was submerged. 5 µL of 6x loading buffer was added to the samples and the whole content

loaded onto the gel. For the determination of the size of the fragments 10µL of 1kb DNA marker was also loaded. By applying a voltage of 90V for approximately 45 min the samples were separated. Afterwards they were visualized and identified by the transilluminator.

For the isolation of a desired DNA fragment from the agarose gel, the gel was carefully laid on an UV-table in order to illuminate the ethidium bromide stained DNA. The desired band was then identified and carefully removed with a scalpel. The gel fragments were purified with the help of the "Invisorb® Spin DNA Extraction Kit" from Invitek according to the manufacturer's protocol.

2.2.5 Cloning PCR Products

Cloning is the process, where a particular DNA- fragment, mostly a PCR product, is inserted into an appropriate plasmid vector and cloning vector, respectively. Afterwards these recombinant plasmids are transformed into competent bacteria cells, which can be used for the amplification of the recombinant plasmids or for the translation of the introduced gene into a protein by its own expression machinery.

One part of this work was to clone the gene of interest - YFV NS1 and the TBE NS1- into an appropriate plasmid vector and to transform these into competent bacteria cells for the expression of YFV and TBE NS1 proteins.

2.2.5.1 Primer Design and Restriction Enzyme Analysis

The gene of interest is amplified through PCR (see chapter 2.2.3.2) using gene specific primer. These primers are flanked with two restriction sites on each end - on the one side for BamHI and on the other side for SalI. This is important for the subsequent ligation step, where the gene of interest and the vector need compatible ends. The sequence of the primer can be found in Chapter A.7.

For cloning the gene of interest into the pTriEx 3 - vector, both the gene of interest and the pTriEx 3 vector have to be cut through restriction enzymes in order to produce complementary ends. Restriction enzymes or restriction endonuclease, which are isolated from bacteria, are able to cut double stranded or single stranded DNA at specific recognition nucleotide sequences through hydrolysis of the DNA phosphodiester bonds. The recognition sequences are called restriction sites. Bacteria use these enzymes as protection against foreign and viral DNA, respectively.

For the digestion, the concentration of the PCR product i.e. the gene of interest was first measured (see chapter 2.2.2) in order to calculate the required concentration of 2µg per digestion. For the PCR products two single digests using the enzymes SalI and BamHI were performed whereas a double digest with the enzymes BamHI and xhoI was performed for the pTriEx 3 vector. Following was assembled in a tube in a total volume of 20µL.

Table 6 Reaction Mixture for Digestion

Components	Volume	Endconcentration
ddH_2O	Up to 20µL	
Buffer O (10X)	2 µL	1x
SalI (10u/µL)	2,4 µL	24U
PCR reaction		2µg
Total	20µL	

This reaction mixture was vortexed, centrifuged and then incubated for 2 hours at 37°C. Afterwards the reaction was purified using the "NucleoSpin® Gel and PCR Clean-Up Kit" from Macherey – Nagel according to the manufacturers protocol. The entire purified DNA was used for the second digestion with the second restriction enzyme. For this, following components were mixed together using the following scheme.

Table 7 Reaction Mixture for the purification after digestion

Components	Volume	Endconcentration
ddH_2O	Up to 20µL	
Buffer BamHI (10X)	2 µL	1x
BamHI (10u/µL)	2,4 µL	24U
PCR reaction		2µg
Total	20µL	

This mixture was again vortexted, centrifuged and then incubated for 2 hours at 37°C. In parallel, the digestion of the pTriEx 3 vector was prepared. For this the following components as shown in Table 8 were mixed together in a total volume of 50µL, vortexed, centrifuged and incubated for 2 hours at 37°C.

Table 8 Reaction Mixture for Digestion

Components	Volume	Endconcentration
ddH_2O	Up to 50µL	
Tango Buffer (10X)	10 µL	2x
BamHI (10u/µL)	4 µL	40U
xhoI (10u/µL)	2 µL	20U
PCR reaction		5µg
Total	50µL	

As control a YF plasmid K3 was used. This was cut with BamHI and SalI and served as insert control and also cut with BamHI and xhoI, which served as vector control.

After incubation, all the digested fragments of each reaction were separated on an agarose gel (see chapter 2.2.4). After separation those fragments with the right size were cut out of the agarose gel and purified with the help of "NucleoSpin® Gel and PCR Clean-Up Kit" from Macherey – Nagel according manufacturer's protocol.

2.2.5.2 Dephosphorylation of Vector

In order to prevent self-ligation of the vector the free phosphate groups at the 5' ends, which result from digestion, have to be removed. This is achieved by the addition of shrimp alkaline phosphatase (SAP), which catalyzes the dephosphorylation of 5' termini DNA.

For dephosphorylation of the vector the following reaction mixture was prepared in a microcentrifuge tube in a total volume of 50µL:

Table 9 Reaction Mixture for dephosphorylation

Components	Volume	Endconcentration
SAP Buffer (10x)	5 µL	1x
SAP	5 µL	24U
PCR reaction		approx. 3µg
Total	50µL	

The tube was vortexed, spun down in a microcentrifuge for 3-5 seconds and incubated for 1 hour at 37°C. Afterwards the mixture is incubated for 15 min at 65°C for SAP inactivation and purified using "NucleoSpin® Gel and PCR Clean-Up Kit" from Macherey – Nagel according to the manufacture's protocol.

2.2.6 Ligation

The insertion of the digested and purified gene of interest into the linearized and dephosphorylated vector is accomplished through the enzyme T4 Ligase. This enzyme forms phosphodiester bonds between the 3' hydroxyl of one nucleotide and the 5' phosphate of another thus ligating the gene of interest into the vector.

For ligation, an insert to vector molar ratio of 3:1 was chosen. The concentrations of insert and vector required for this ratio were calculated according following formulae:

$$x\ [ng\ Insert] = \frac{3\ x\ bp\ Insert\ x\ ng\ vector}{bp\ vector}$$

Next, the following reaction mixture was prepared in a microcentrifuge tube in a total volume of 20µL:

Table 10: Ligation Mixture

Components	Volume	End-concentration
ddH_2O	Up to 20µL	
Ligation Buffer (5X)	2 µL	1x
T4 Ligase	1 µL	
Insert (3x molar mass)	x	x ng
Vector pTriEx3 (lineraised)	y	50ng
Total	50µL	

The tube was vortexed, briefly spun down in a microcentrifuge and incubated on ice overnight. Subsequently, the recombinant plasmids are transformed into competent bacteria cells (see chapter 2.3.1).

2.3 Microbiological methods

2.3.1 Heat-shock Transformation into competent bacteria cells

Transformation is the process by which bacterial cells take up foreign DNA molecules. These bacterial cells are able to replicate the foreign gene construct by using their own replication machinery. However, not all bacterial strains are capable to incorporate foreign DNA. Hence, they have to be treated with special buffers prior transformation to make them competent i.e. to make them capable for the intake of foreign DNA. These buffers contain salts such as rubidium chloride, calcium chloride or cobalt chloride among other chemicals. The salt solution forms crystals through the bacterial cell membrane. These crystals dissolve when the bacterial cells are being heat shocked for 45 seconds at 43.5°C and small pores are formed momentarily, through which the foreign DNA can enter passively. After transformation the bacterial cells are incubated for 1-2 hours at 37°C in a shaker and afterwards plated onto LB-plates [83]. In order to select for recombinant colonies all cells are plated on selective media. Usually the foreign DNA fragment carries a selection marker in the form of an antibiotic resistance gene, so that only transformed cells will be able to grow on these plates.

2.3.1.1 Transformation of recombinant pTriEx 3 into One Shot® TOP10 Competent Cells

This transformation was performed for the clonal amplification of the recombinant vector. Briefly, One Shot® Top10 competent cells were thawed up on ice and 20µL of the ligation preparation were added to it. This mixture was then swirled gently and incubated for 30 minutes on ice. After that, the transformation mix was heat-pulsed in a 42°C water bath for 30 sec and again incubated for 2 min on ice. Two hundred fifty µL of prewarmed SOC medium was added and the cells were incubated in a shaker at 225 rpm for 1 hour at 37°C. Subsequently, 100µL and 200µL of transformed Top10 competent cells were spread on LB- ampicillin agar plates and incubated upside-down over night at 37°C. In order to screen for positive clones a colony PCR was performed (see chapter 2.2.3.3).

2.3.1.2 Transformation of recombinant pTriEx 3 into RosettaBlue™ (DE3) pLacI Competent Cells

For the expression of the NS1 protein, pTriEx 3-NS1 clones were transformed into RosettaBlue™ (DE3) pLacI competent cells.

For the transformation 20µL of RosettaBlue™ (DE3) pLacI competent cells were thawed up on ice and 1µL of the recombinant plasmid DNA (pTriEx 3-NS1) was added. This mixture was

then swirled gently and incubated for 5 min on ice. After that, the transformation mix was heat-pulsed in a 42°C water bath for 30 sec and again incubated for 2 min on ice. Eighty µL of prewarmed SOC medium was added to each transformation mix and these were then incubated in a shaker at 250 rpm for 1 hour at 37°C. Subsequently, 20µL, 30µL and 50µL of transformed competent cells were spread on agar plates containing ampicillin (80µg/mL), chloramphenicol (30µg/mL) as selection marker and glucose (1%) and incubated upside-down over night at 37°C. In order to screen for positive clones, bacterial colonies were picked, inoculated into 2 mL of LB medium containing the appropriate selective antibiotic and grown with vigorous shaking overnight. Afterwards the DNA was isolated using the "Invisorb®Spin Plasmid Mini Kit Two" from Invitek according to the manufacturer's protocol (see following chapter 2.3.2).

2.3.2 Preparation of plasmid DNA on small scale

For the isolation of plasmid DNA on small scale the "Invisorb®Spin Plasmid Mini Kit Two" from Invitek and the "NucleoSpin® Plasmid" DNA purification kit from Macherey-Nagel was used according to the manufacturer's protocol. For both the kits the isolation of the plasmids is based on the principle of alkaline lysis.

For the plasmid preparation, a single bacterial colony had been inoculated into 2 mL of LB medium containing the appropriate selective antibiotic and grown with vigorous shaking overnight. The overnight culture was then transferred into a 2.0 mL microcentrifuge tube and centrifuged for 1 min or 30 sec at maximum speed to pellet the cells. The supernatant was removed as completely as possible and the cell pellet was resuspended in 250 µL of Solution A/Buffer A1 by vortexing the tube. The lysis of the cells was initiated by adding 250 µL Solution B/Buffer A2 thereby swinging the tube carefully. Subsequently, 250µL Solution C/300µL Buffer A3 was added, the preparation was mixed gently and thoroughly by shaking the tube 5 times and afterwards centrifuged for 5 – 10 min at full speed (12,000 – 14,000 x g). The clarified supernatant containing the plasmids was transferred into a spin filter, incubated for 1 min so that the plasmid DNA could be adsorbed by the filter membrane. Digested RNA, cellular proteins and metabolites remained in the lysate and were drawn through by centrifuging the tube with the spin filter for 1 min at 8,000 x g. The filtrate was discarded and residual contaminants were washed away by using 600µL/750µL Wash Solution. The microcentrifuge tube with the spin filter was again centrifuged for 1 min at 8,000 x g and the filtrate discarded. For the complete removal of residual ethanol, the tube with the spin filter was centrifuged again for 3 min at full speed (12,000 – 14,000 x g). Afterwards 50µL of aqua dest. was added directly onto the center of the spin filter surface and this was incubated for 10 min at room temperature.

Finally, the plasmid DNA was eluted by centrifuging the tube at 8,000 x g for 1 min and stored at -20°C.

2.3.3 Preparation of plasmid DNA on large scale

For the isolation of plasmid DNA on large scale the "Invisorb® Plasmid Maxi Kit" from Invitek was used according to the manufacturer's protocol. The isolation of the plasmids is based on the principle of alkaline lysis.

For the plasmid preparation, a single bacterial colony had been inoculated into 100 mL of LB medium containing the appropriate selective antibiotic and grown with vigorous shaking overnight. The overnight culture was then transferred into two 50 mL tubes and centrifuged for 15 min at 1,750 x g to pellet the cells. The supernatant was removed as completely as possible, the cell pellet of each tube was resuspended in 8 mL of Solution I by vigorous vortexing and 100µL RNase/Solution I mixture was added. The lysis of the cells was initiated by adding 12 mL Solution II thereby swinging the tube carefully and incubating it for 5 min at room temperature. Subsequently, 12 mL Solution III was added, the preparation was mixed gently and thoroughly by shaking the tube 5 times and afterwards placed on ice for 5 min. After the incubation period, the falcon tubes were inverted two times and then centrifuged for 10 min at minimum 1,750 x g. The clarified supernatant containing the plasmids was transferred into syringe with syringe prefilter, the solution was filtered into a new receiver tube and 12 mL Binding Solution PL was added. The tubes were then inverted two times. A maximum of 12 mL of this solution was loaded into a spin filter afterwards and centrifuged at 1,750 x g for 10 min. The filtrate was discarded and residual contaminants were washed away by using 15 mL Wash Buffer PL. The falcon tube with the spin filter was again centrifuged at 1,750 x g for 10 min. This step was repeated until all Wash Buffer PL had passed the filter. For the complete removal of residual Wash Buffer PL the tube was centrifuged again for 20 min at full speed 1,750 x g. Afterwards 1 mL of aqua dest. water was added directly onto the center of the spin filter surface and this was incubated for 10 min at room temperature. Finally, the plasmid DNA was eluted by centrifuging the tube at 1,750 x g for 5 min and stored at -20°C.

2.4 Protein Biochemical Methods

2.4.1 Induction of Proteinexpression

NS1 expression in RosettaBlue™ (DE3) pLacI was induced by IPTG. For this purpose, one positive clone carrying the vector with the gene of interest was cultured in 3mL of fresh LB

media (LB-amp-cm-glc) containing ampicillin (80µg/mL), chloramphenicol (30µg/mL) and glucose (1%) and incubated over night at 30°C with shaking at 200 rpm. The next morning, the starter culture was diluted 1:20 with fresh LB-amp-cm-glc medium (125µL of starter culture + 2,375mL medium) and incubated for 2h at 37°C with shaking at 200 rpm. Afterwards the optical density was measured every 15 min till the bacterial cultures reached an OD of 1.2. Once this was reached the culture was split into two test tubes and again diluted 1:2 with fresh LB-amp-cm-glc medium (2.5 mL of culture + 2.5 mL of media), so that the OD was reduced to a value of 0.6. One of the diluted cultures was then induced with 1 mM IPTG, i.e. 50µL of 0.1 M IPTG stock solution was added to the culture. For the other test tube containing the diluted culture no IPTG was added as this served as non-induced control. Both the cultures were then incubated at 37°C with shaking at 200 rpm. After 1h and 4h 2mL fractions were taken and centrifuged at highest speed for 1 min. The supernatant was decanted in another tube and both supernatant and pellet were frozen at -20°C.

2.4.2 Protein purification using BugBuster™ Protein Extraction Reagent

The frozen cell pellets were thawed, weighed and then resuspended in 5mL of room temperature BugBuster™ reagent for every one gram of cell pellet. To the resuspended cells 1µL per mL of Benzonase and 1mL per 10g cells of protease inhibitor (Protease Inhibitor Cocktail Set III) were added and incubated for 20 min at room temperature with shaking. Benzonase removes nucleic acid contaminants and protease inhibitors protect the protein of interest from degradation. After incubation cells were centrifuged at 16,000 x g for 20 min and 4°C to remove the insoluble cell debris. The supernatant, which contained the soluble extracts, was transferred to fresh tubes and frozen at -20°C till analysis. The pellet was further processed for inclusion body purification. For this, the same volume of BugBuster™ reagent that was used initially was used for resuspending the pellet from the last step. To that 200µg/mL of lysozyme was added, mixed by vortexing and incubated for 5 min at room temperature. Afterwards 6 volumes of 1:10 diluted BugBuster™ reagent was added to the suspension and vortexed for 1 min. Subsequently, the suspension was again centrifuged at 16,000 x g for 15 min at 4°C to collect the inclusion bodies. The supernatant was carefully removed and the pellet containing the inclusion bodies was again resuspended in 0.5 volumes of 1:10 diluted BugBuster™ reagent. This step was repeated 2 more times. The final pellet was then resuspended in laemmli buffer, which was 1:5 diluted with PBS and frozen at -20°C.

2.4.3 SDS-Gelelectrophoresis

The purified proteins are separated by an SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Hereby the proteins are incubated with Sodium dodecyl sulphate (SDS) and a disulphide bonds reducing agent. SDS is an anionic detergent, which applies a negative charge to each protein in proportion to its mass and also denatures them. Through the addition of a reducing agent like β-mercaptoethanol, which cleaves any disulphide bonds, and the denaturing detergent SDS the proteins can be totally unfolded. The negatively charged protein molecules migrate through a matrix with an electric field according their size.

For the separation of the proteins a discontinuous SDS-PAGE was employed. In a discontinuous system, a so called non-restrictive large pore stacking gel is layered on top of a running gel also called resolving gel. Both these gels are made with different buffers and hence have different pH values, which leads to the proteins concentrating into narrow zones in the stacking gel and destacking or resolving afterwards in the small pore resolving gel. The advantage of such a system is that the resolution is much greater than that obtained with other gel systems. In this work, precast Thermo Scientific™ Precise™ Tris-Glycine gels in a concentration of 12% were used.

The purified proteins, which have been suspended in laemmli buffer after purification, were thawed, 250µL ß-Mercaptoethanol per 1mL added to each sample and then heated for 5 min at 95°C. Electrophoresis buffer (1xTGL) was then poured into the electrophoresis chamber, the ready-to-use gel was carefully inserted and 50µL of each sample was cautiously loaded on the gel. In addition, a protein marker was also loaded, so that the respective proteins can be identified later on. The gel was run at 90 V at first until the proteins reached the running gel. At this time, the voltage was increased to 145 V. The gel was stopped when the dye front reached the bottom of the gel (approx. after 45 min) and subjected to Western Blotting (see Fig. 8).

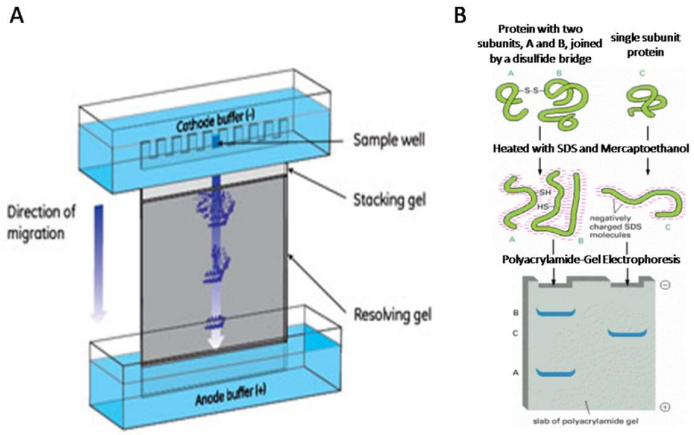


Fig. 8 SDS-polyacrylamide gel electrophoresis
A) Composition of an SDS-PAGE B) Proteins are unfolded and denatured by the treatment with SDS, ß-Mercaptoethanol and heat. SDS also applies a negative charge to each protein in proportion to its mass. The negatively charged proteins migrate through the matrix with an electric field according their size [84, 85]

2.4.4 Transfer and Blotting

In this step, the separated proteins are transferred from the gel onto a membrane. The transfer is accomplished through electric current. For this the membrane is first activated in methanol for 15 min and afterwards equilibrated in cold 1x transfer buffer together with 4 sheets of Whatman paper for another 15 min. The SDS polyacrylamide gel is first soaked in aqua dest. for 15 min and then also equilibrated in 1 x transfer buffer for 15 min. Afterwards the blot was assembled as depicted in Fig. 9. Blotting occurred at a current of 230 mA 1h for each gel.

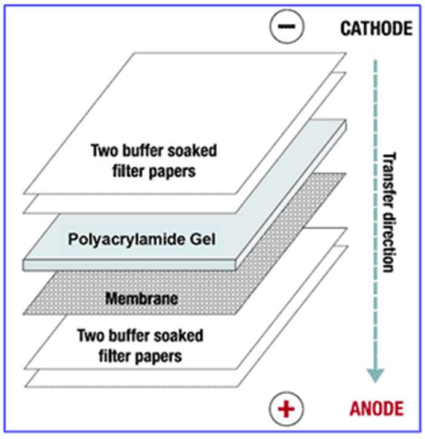


Fig. 9 Assembly of a Semi Dry Blot
The migration of the proteins from gel to the membrane is caused by electric current [86]

After the transfer of the proteins, the PVDF membrane is stained with Coomassie in order to check the effectiveness of the transfer. Afterwards the membrane is completely de-stained and washed with TBST.

2.4.5 Immunodecoration

To reduce non-specific protein interactions between the membrane and the antibody the membrane is blocked for one hour with Blocking Solution. This was followed by the incubation of the membrane with the primary antibody, which was diluted in 0.5% Blocking Solution for 1 hour at room temperature and with shaking. As primary antibody, a 1:1000 diluted α-His antibody was applied. This first antibody binds specifically to the his-tag of the proteins of interest and should not bind to any of the other proteins on the membrane. After 1 hour the membranes were washed 5 times for 5 min with PBS-T (0.1% Tween) and then incubated with the second antibody, α-Rabbit-HRP, 1:x diluted in 0.5% Blocking Solution for 45 min at room temperature. The antibody binds non-specifically to the primary antibody and thereby generates antigen-antibody-antibody complexes. Typically, the secondary antibody is conjugated to a reporter enzyme, which in our case is horseradish peroxidase. Horseradish peroxidase catalyzes the degradation of an enhanced chemiluminescent substrate (ECL) that results in the emission of light. For chemiluminescent detection, the membrane has to be washed again as described above and equal volumes of the ECl substrate have to be added. Detection was achieved by exposing the blot to X-ray film.

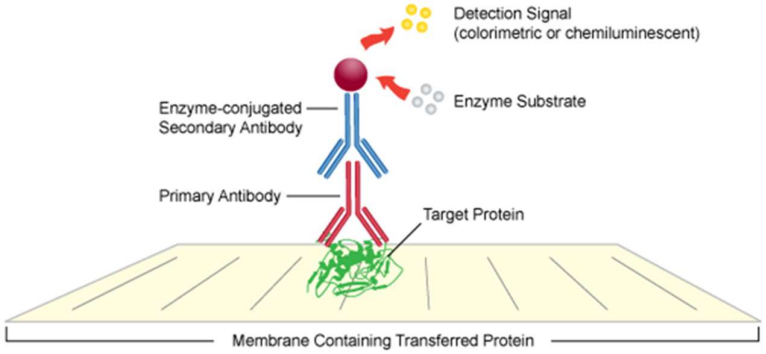


Fig. 10 Detection in Western Blots
The target proteins, which are immobilized onto the membrane are detected by the interaction between target proteins, primary and secondary antibodies. The secondary antibody is conjugated to HRP, which when substrate is added results in the emission of light [87]

2.5 Immunological Methods

2.5.1 Indirect Immunofluorescence Assay

An indirect immunofluorescence assay (IFA) was performed for the characterization of serum samples of YF vaccinees in regard to their IgM levels. Once characterized these serum samples can be used for the establishment of an enzyme-linked immunosorbent assay for antibodies against YFV.

The IFA was performed using a commercial slide from Euroimmun AG. These slides contain Biochips, which are millimeter-sized cover glasses coated with YFV - infected Vero cells. If the serum samples contain antibodies against YFV, these will bind to the antigens presented by the infected cells on the biochips. These bound antibodies can then be captured in a second step by a secondary antibody (anti-human), which is conjugated to Fluorescein. This complex can then be visualized by a fluorescence microscope.

Briefly to the protocol, a reagent tray is thoroughly rinsed with water and the BIOCHIP slides are incubated at room temperature. Serum samples then are diluted 1:10 with PBS. For IgM detection, diluted serum samples are incubated with an RF absorbent for 15 min at room temperature. Thereby IgG antibodies and rheumatic factors are removed from the serum. Afterwards samples are centrifuged at 200 rpm for 5 min. In case of IgG detection this step is not required. Subsequently, 30µL of diluted serum samples are applied to each reaction field of the reaction tray. The BIOCHIP slides, which should have reached room temperature, are then fitted into the corresponding recesses of the reaction tray and incubated for 30 min at room temperature. Afterwards BIOCHIP slides are washed with a flush of PBS-Tween and then immediately immersed in a cuvette containing PBS-Tween for at least 5 min. 25µL of fluorescein-labelled anti–human IgM (conjugate) is then applied to each reaction field of a new reaction tray and the washed BIOCHIP slides are again fitted into the corresponding recesses of the reaction tray. This is incubated for 30 min at room temperature. After incubation, the BIOCHIP slides are washed again as described. In the meantime, drops of glycerol/PBS are placed onto a cover glass. The BIOCHIP slides are removed from the PBS-Tween, carefully dried with a paper towel and put onto the cover glass facing downwards. The slides were then observed with a fluorescence microscope.

2.5.2 Enzyme-linked Immunosorbent Assay

An enzyme-linked immunosorbent assay (ELISA) is a very common laboratory technique used for the detection and quantification of specific antibodies or antigens in any sample. The basic principle of the method is the specific linkage of an antigen (Indirect ELISA Fig. 11) or antibody (Sandwich ELISA Fig. 11) coated on a solid surface, mostly multi-well plates, to its target antibodies or antigens in a sample. The captured antibodies or antigens can then be detected by a primary unlabeled antibody in conjunction with an enzyme-linked secondary antibody. By the addition of an enzymatic substrate a change in color is caused, which is proportional to the amount of antigen or antibody in the sample (Fig. 11).

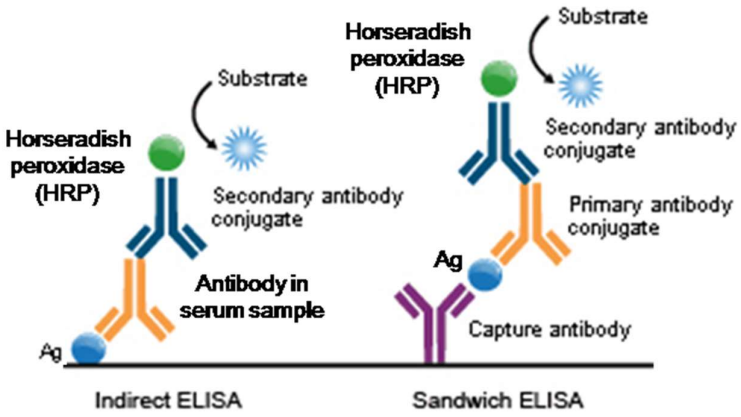


Fig. 11 Two different ELISA formats. The scheme on the left-hand side shows the principle of an indirect ELISA. Here, wells are coated with an antigen to which antibodies in serum samples bind. The bound antibody is then captured by a secondary antibody conjugated to an enzyme (horseradish peroxidase). The detection of this complex takes place by the addition of an enzymatic substrate, which is catalyzed by the conjugated enzyme. The thus produced chemiluminescent signal is then captured in the last step. On the right-hand side, the principle of a sandwich ELISA is shown. The difference here is, that wells are coated with an antibody, which is captured by antigens in the samples. These are then detected by a primary antibody against this antigen and an enzyme-linked secondary antibody. The addition of a substrate leads to a change in color, which is again captured in a last step [88]

In order to test serum samples of YFV vaccinees for IgG and IgM antibody levels, the aim here was to establish an ELISA. For this purpose, different parameters like optimal buffer solutions, optimal antigens and different controls (positive and negative) had to be first identified. All antigens used in this assay are presented in the following table. All recombinant proteins (parts of the E-III domain of the E protein, the whole E protein as well as the NS1 protein) used in this assay have been provided by the company NovaTec GmbH.

Table 11 Different YFV antigens
These antigens were used for coating wells of a 96-well plate so that the antigen giving the best results can be chosen.

Antigens	Coating concentration/dilution
Cell lysate of YFV infected cells	1:50 diluted
Supernatant 17D	1:10 diluted
Supernatant Asibi	1:10 diluted
E-III domain	10µg/mL
Recombinant E protein	10µg/mL
E3 pp40 (E-III domain)	10µg/mL
E3 pet44 (E-III domain)	10µg/mL
E-full 4T1	10µg/mL
YFV wildtype infected mouse brain	1:100 diluted
Recombinant NS1 protein	1µg/mL

The protocol, which was chosen at the end giving the best result is as follows:

The antigen was diluted in carbonate buffer and 100µL of this solution was given into each well of a microtiter plate. The plates were then covered and incubated over night at 4°C. The next morning the antigen solution was removed and the plates were washed with 300µL washing buffer (PBS-Tween 0.05%) per well. The washing buffer solution was removed over a sink and the remaining unsaturated surface-binding sites were blocked by adding 150µL of blocking solution to each well. Subsequently plates were incubated for 1h at room temperature. In the meantime, serum samples were diluted 1:100 with dilution buffer. After 1h of incubation, the blocking solution was removed over a sink and 100µL of diluted serum sample was applied to each well of the microtiter plate. This was again incubated for 1h at 37°C. After 1h, wells were washed 3 times with 300µL washing buffer per well. Subsequently, 100µL of 1:5000 diluted conjugated secondary antibody (α-human-IgG-HRP or α-human-IgM-HRP) was added to each well and incubated for 30 min at room temperature in the dark. The wells were washed again as described above and 100µL of substrate solution (TMB) was dispensed per well. After sufficient color development (10 to 15 min) the reaction was stopped by the addition of 100µL

of stop solution per well. The optical density (OD) per well was measured at 450 nm with 620 nm as reference wavelength using an ELISA microplate reader from Tecan.

We also obtained microtiter plates, which were already coated with anti-μ chain specific antibodies (specific to human IgM antibodies). These plates were also tested according to a similar protocol as described above. Briefly, wells were incubated with diluted serum samples for 1h at 37°C, washed with washing buffer and then incubated again with YF-17D virus solution (1:10 diluted in PBS) for 1h at room temperature. Afterwards wells were washed again 3 times with 300μL washing buffer per well, carefully dried and then incubated with a biotinylated monoclonal antibody (mAb 6330 biotinylated) against YFV-17D for 1h at room temperature. Subsequently, cells were washed as described above and incubated with HRP conjugated streptavidin for 30 min at room temperature in the dark. After incubation wells were washed again and incubated with 100μL of substrate solution (TMB) per well. After sufficient color development (10 to 15 min) the reaction was stopped by the addition of 100μL of stop solution per well. The optical density (OD) per well was measured at 450 nm with 620 nm as reference wavelength using an ELISA microplate reader from Tecan.

Each run contained a positive and negative control. Serum samples were used as positive and negative controls. These have been repeatedly positive and negative in several previous assay runs. Furthermore, wells without antigens and samples were included in each run. These so called blank wells represent the background signal resulting from reagents and the microtiter plate.

2.6 Virological Methods

In this chapter, the protocols for determining the quantity of virus in a viral solution as well as for determining neutralizing antibodies in a serum sample are presented. The principles of the assays are described in the following.

The gold standard for determining the quantity of infectious virus in a viral solution is the plaque assay. This method helps to determine the amount of infectious virus particles in a virus solution as plaque forming units per ml (PFU/ml). The technique is based on the ability of a single infectious virus to form a “plaque”, i.e. a spot of dead cells, on a confluent monolayer culture of cells [89].

In a plaque assay, different dilutions of a virus stock are inoculated onto susceptible cell monolayers. After a specific time of incubation, which is necessary for the cells as they have to

adhere to the plate surface, the cells are overlaid with a viscose solution. This solution consists mostly of agar/agarose or carboxy-methyl- cellulose (CMC) and prevents the virus to spread through diffusion. Hence, only neighboring cells can be infected by the virus. Upon infection, the virus starts to replicate using the host cell's machinery. The result are morphological and biochemical changes, which are called cytopathic effects. Cells which show these effects can be easily differentiated from healthy cells under the microscope as they have specific forms like cell rounding, cell fusion or total cell lysis. As more and more neighboring cells get infected by the single virus, small circular zones become visible, where cells have died because of cell lysis. These circular zones can be seen as plaques and detected by various staining methods. Only viable cells are stained whereas dead cells are not stained appearing as un-stained spots against the colored background. To determine the plaque forming units per ml (PFU/ml) the plaques are enumerated under a microscope, the plaque counts are averaged over wells and the average is then divided by the dilution times the volume [89]. Fig. 12 depicts the scheme of a plaque assay.

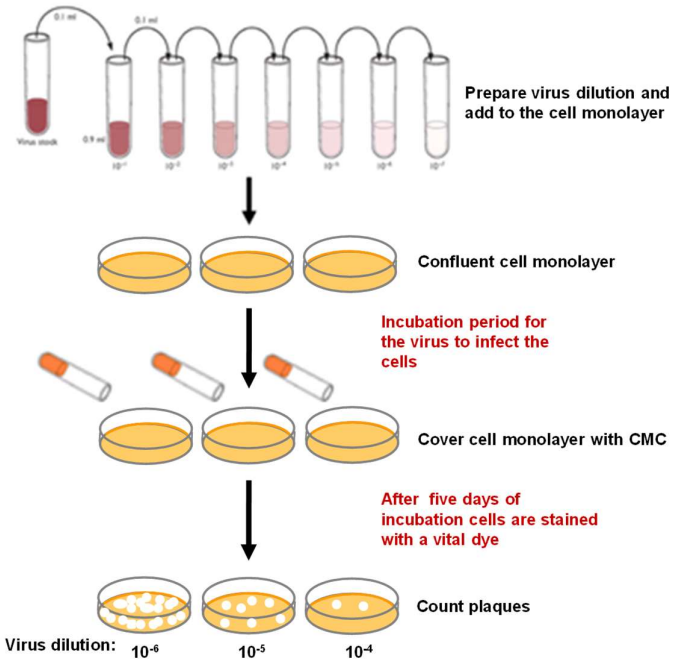


Fig. 12 Scheme of a plaque assay
Decadal serial dilutions of a virus stock are prepared and inoculated with confluent cells. After a certain infection period, the cell monolayer is covered with CMC. This step is necessary so that the released virus particles do not spread through diffusion to adjacent cells. The cells are incubated for five to seven days depending on the cell type. After incubation, cells are washed and stained with a vital dye (e.g. naphthalin blue).

The gold standard for detecting neutralizing antibodies in a serum sample is a serological method called the plaque reduction neutralization assay [90-92].

The human body's immune system responds to a viral infection by producing antibodies, amongst other factors, against many epitopes on multiple virus proteins. Some of these antibodies, for example like IgG and IgM, are able to neutralize the virus by inhibiting further infection of more cells. The process of viral neutralization can be accomplished by a number of ways, which are inhibition of the uptake of virions into cells, prevention of virion binding to receptors and uncoating of the genomes in endosomes or aggregation of virus particles. This various ways of virus neutralization are depicted in Fig. 13 [90].

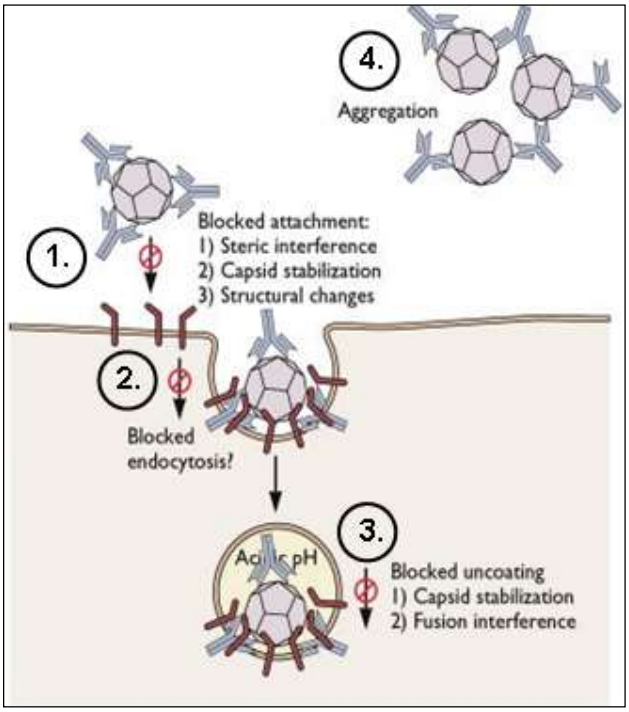


Fig. 13 Process of viral neutralization
Neutralizing antibodies are able to neutralize the virus through different ways: 1) They either inhibit binding of the virus to host receptors, 2) or the virus can indeed bind to host receptors, but the following uptake i.e. endocytosis of the virions is prevented, 3) another way of neutralization is to block encapsidation of the viral genome, 4) or the aggregation of virus particles, so that they become inoperable [93].

Neutralizing antibodies remain for a very long time and mediate permanent protection against a re-infection. Besides neutralizing antibodies also non-neutralizing antibodies are produced after an infection. The difference between these two antibodies is that non-neutralizing antibodies can bind virions, indeed, but they cannot neutralize them, i.e. neutralize their infectivity [90].

The properties of neutralizing antibodies are detected in the plaque assay by incubating a defined concentration of virus particles with a serum sample for an appropriate time. The incubation time is required for the recognition of antibodies and antigens and their binding to form antibody and antigen complexes. The serum-virus solution is then inoculated into susceptible cells. In case of positive serum, the neutralizing antibodies are able to recognize and bind the virions and neutralize them, so that the virus loses the infectious properties. Hence, the cells show no cytopathic effects, i.e. no formation of plaques. In case of a negative reaction, the cells would show formation of plaques as the serum does not contain neutralizing antibodies which could inhibit virus replication within the cells [94].

The microneutralization assay is based on the same principle as the plaque reduction neutralization assay with only the difference that it is performed in a 96-well plate. Furthermore, the evaluation of the assay is based on the presence of cytopathic effects in the infected cells. Hence, here the evaluation relies on a yes or no approach and not on counting plaques [94].

2.6.1 Viral titration by plaque assay

For the quantification of the virus titer, 200µL of a PS cell suspension at a density of $6x10^5$ cells/ mL in L15 medium were seeded in a 24 well plate. The virus sample was serially diluted and 200µL of each virus dilution was given to the cells in triplicate. For the negative control only medium was given to the cells instead of the virus dilution. For an even cell and virus distribution the plate was gently rocked back and forth and then side to side and then incubated for 4 hours. After 4 hours, the cells have attached to the ground of the plate so that 400µL of a viscose CMC solution could be poured carefully over the virus-cell solution to avoid virus spread. The infected PS cells were cultured in L15 with 10% FCS at 37°C in a humidified environment without CO_2 for 4 days. Four days post infection the medium was removed, cells washed with diluent solution and the monolayer fixed with 3.7% formaldehyde. Formaldehydes introduce cross-links between reactive groups of different cell components as for example proteins, nucleic acid and lipids. After 15 min, the formaldehyde solution was removed and the cell monolayer was stained with naphthalene black solution for 30 min. The cells were washed with water and kept for drying. Afterwards plaques were readily visible by eye and could be counted directly. The counted plaques were then transformed according following formula to infectious units per milliliter virus solution (plaque forming units; pfu) [90].

$$\boldsymbol{pfu/mL = \frac{n_p x d}{V}}$$

Where n_p denotes the counted plaques, d the dilution factor and V the volume of the virus solution.

2.6.2 Viral titration by $TCID_{50}$

Not all viruses are able to form plaques, hence a different method than the plaque assay to determine virus titer in a virus solution is needed. Another way to measure the concentration of viruses in a sample is the endpoint dilution assay, where typically the 50% tissue culture infectious dose – the $TCID_{50}$ value – i.e. the virus concentration producing a cytopathic effect in 50% of the cultures inoculated, is calculated.

The endpoint dilution assay is usually performed in a 96-well plate. The principle of this assay is similar to the plaque assay. The only difference is that this assay is a quantal assay whereas the plaque assay is a focal assay. Focal assays are based on counting so called foci or plaques, which result from the infection of one single infectious particle. This permits a quantitative determination of the number of infectious units. The quantal assay on the other hand permits a qualitative determination. It uses the "all or none" approach, which means that each well is observed under a microscope if the cells show a cytopathic effect or not. Hence, in this assay only the presence of infectious virus is detected.

For this assay, 45µL of DMEM medium were put in each well of a 96- well plate. The virus was serially diluted with 5µL pipetting from one well to the other. Each dilution was applied in quadruplicate, as only when using several repetitions, the method becomes more accurate. After serially diluting the virus directly in the wells, 150µL of PS-cells at a density of $4x10^5$ cells/ mL in DMEM medium or 50µL of Vero E6 cells at a density of $4x10^5$ cells/ mL also in DMEM medium were added to each well. The infected PS cells were cultured at 37°C in a humidified environment without CO_2 for 5 days, whereas the infected Vero E6 cells were cultured at 37°C in a humidified environment with CO_2 for 7 days. Afterwards cells were washed with diluent solution and the monolayer was fixed with 3.7% formaldehyde. After 15 min, the formaldehyde solution was removed and the cell monolayer was stained with naphthalene black solution for 30 min. The cells were washed with water and kept for drying. Afterwards each well was observed under the microscope to see in which well the cells showed cytopathic effects. The $TCID_{50}$ value was calculated after the formulae of Reed and Muench [94] and also Spearman and Kärber [94]. Both the formulae are depicted below:

Reed and Muench:

$$\boldsymbol{I = \frac{p_{well}^{>50\%} - 50\%}{p_{well}^{>50\%} - p_{well}^{<50\%}}}$$

Where *I* denotes the interpolated value of the 50% endpoint (also known as the proportional distance), $p_{well}^{>50\%}$ the percentage of wells infected at dilution immediately above 50% and $p_{well}^{<50\%}$ the percentage of wells infected at dilution immediately below 50%.

The $TCID_{50}$ value can be calculated with the value of the proportional distance after following formula:

$$\boldsymbol{TCID_{50} = 10^{log(d>50\%) - (I x log\ h)}}$$

where $log\ d^{>50\%}$ denotes the log of the virus dilution above 50%, *I* the proportional distance and *h* the dilution factor.

Spearman and Kärber:

$$\boldsymbol{TCID_{50} = \frac{d^{100\%} + \frac{1}{2} - n_{tot}^{tu}}{n^{tud}}}$$

where $d^{100\%}$ denotes the highest dilution giving 100% CPE, n_{tot}^{tu} the total number of test units showing CPE and n^{tud} the number of test units per dilution.

2.6.3 Plaque reduction neutralization assay

The first step of the plaque reduction neutralization assay was to prepare serial dilutions of serum samples in L15-medium with 10% FCS, 1% Glutamine, Streptomycin and Penicillin. The serum dilutions were challenged with an equal volume of the Vero B4 17D virus, which had been previously titrated to give 20 PFU per 200 µl of inoculum. The serum-virus solutions were then heat-inactivated at 56°C for one hour, in order to destroy complement factors. In the meantime, 200µL of a PS cell suspension at a density of $6x10^5$ cells/ mL in L15 medium was seeded in a 24 well plate. After one hour, 200µL of the serum-virus solution was given to the cells in triplicates. For the cell control only medium was given to the cells instead of the virus dilution and for the virus control only virus without serum was added. Furthermore, a negative serum, from a person who had not been infected, was tested in parallel.

For an even cell and serum-virus distribution the plate was gently rocked back and forth and then side to side and then incubated for 4 hours. After 4 hours, 400µL of a viscose CMC solution could be poured carefully over the serum-virus-cell solution to avoid virus spread. The infected PS cells were cultured in L15 with 10% FCS at 37°C in a humidified environment without CO_2 for 4 days. Four days post infection the medium was removed, cells washed with diluent solution and the monolayer fixed with 3.7% formaldehyde. After 15 min, the formaldehyde solution was removed and the cell monolayer was stained with naphthalene black solution for 30 min. The cells were washed with water and kept for drying. Afterwards plaques were readily visible by eye and could be counted directly. The neutralizing antibody titer could be calculated after the formulae of Reed and Muench.

2.6.4 Microneutralization assay

The microneutralization assay is based on the same principle as the plaque reduction neutralization assay. The only difference is that this assay is performed in a 96-well plate and that here the concentration of neutralizing antibodies, which are able to neutralize 50% of the virus concentration inoculated into cells, is determined.

The first step of this assay was the heat-inactivation of all serum samples at 56°C for 30 min. Then two-fold dilutions of each serum sample were prepared with medium in 96-well plates in a total volume of 25µL for each well to obtain dilutions of 1:4 to 1:256. These serum dilutions were challenged with an equal volume of the Vero B4 17D virus, which had been titrated previously to give 100 $TCID_{50}$ per 25 µl of inoculum. The serum-virus solutions were then incubated at 37°C for one hour in a 5% carbon dioxide, 90% humidity atmosphere. After one hour, 100µL of a PS cell suspension containing $6x10^5$ cells/ mL in Dulbecco's modified Eagle medium (DMEM) was added into each well. The medium was supplemented with FCS so that each well contained a final FSC concentration of 5% at the end. All serum samples were tested in duplicates.

Each plate must include different controls. To verify the amount of virus actually used for the test and to see how good the activity of the virus is, a virus control was employed. For this, the virus dose was diluted with medium to 10, 1 and 0.1 $TCID_{50}$/25µL. From each dilution, 25µL was placed in four wells respectively, 75µL media added and finally 100µL of the cell suspension was given in the same way as for the serum under test. The second control is the serum control, which is necessary to exclude any toxic effects of the serum on the cell. For this control, 25 µL of serum was incubated with 25µL of medium for one hour at 37°C and afterwards 100µL of cell suspension was added in the same way as for the serum under test.

The last control is the cell control, which just contains 100µL of the cell suspension with 100 µL medium. This control serves to check the condition of the cells. Furthermore, a negative serum from a person who has not been infected is tested in parallel under the same conditions as the serum to be tested.

For an even cell-and-serum virus distribution the plate was gently rocked back and forth and then side to side and then incubated for five days at 37°C in a 5% carbon dioxide, 90% humidity atmosphere. After five days the medium was removed, cells were washed with diluent solution and the monolayer fixed with 3.7% formaldehyde. After 15 min, the formaldehyde solution was removed and the cell monolayer was stained with naphthalene black solution for 30 min. The cells were washed with water and kept for drying. Afterwards plates were evaluated and each well was observed under a microscope for signs of cytopathic effects in the infected cells. The serum dilution, which prevents 50% of replicate inoculation (ie, in which 1 of 2 duplicate infections is blocked), is determined as the neutralization titer (*NT*). Whenever infection is prevented in both duplicate wells (100%) at a particular dilution and present in both duplicates (100%) at the next dilution, the *NT* is determined as the geometric mean of the 2 dilutions. If complete infection is observed at all serum dilutions, the *NT* is determined as <1:4 the starting serum dilution.
The same assay was performed with Vero E6 cells under the same conditions. The only difference was that a lower concentration of $1.3x10^5$ cells/ mL was needed.

Both the PS and the Vero E6 cells were cultured in DMEM with 10% FCS, 1% Glutamine, Streptomycin and Penicillin in a humidified environment with CO_2 for 5 days.

2.7 The xCELLigence™ system - Cellular Impedance Measurement

The Real-Time Cell Analyzer (RTCA) system, xCELLigence™, is a novel tool based on microelectronic technology and biological expertise. It allows monitoring cellular processes, as for example cell growth, cell proliferation, cell adhesion and morphological changes in real time. One further advantage is that the cells do not have to be labelled for the experiments [95].

The xCELLigence™ system consists of the RTCA, the RTCA SP station, the RTCA laptop with integrated software and last but not least of a tissue culture plate, a so-called E-Plate. This E-plate is of particular importance for this system, as it contains small micro-electrodes, which are integrated into the bottom of the plates (the letter “E” stands for electronic plate) [95].

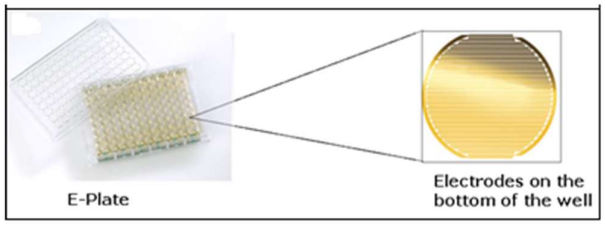


Fig. 14 The E-plate of the xCELLigence™ system
This picture shows the micro-electrodes on the bottom of each well of an E-plate [95].

The basic principle of the detection system relies on the measurement of electrical impedance changes resulting from the cells interaction with the microelectrodes. The electrical impedance, also simply called impedance, is the resistance of a circuit to alternating current.

The microelectrodes react to the change of the local ionic environment at the electrode/solution interface in the presence of cells. This leads to an increase in the impedance (Z). With more cells attaching to the microelectrodes at the bottom of the culture plates the increase in the impedance becomes larger. The impedance will be zero, if cells are not strongly attached or present in the wells.

Furthermore, increased cell adhesion and spreading also leads to a larger change in electrode impedance. Thus, impedance depends on the quality of the cell interaction with the electrodes as well [95].

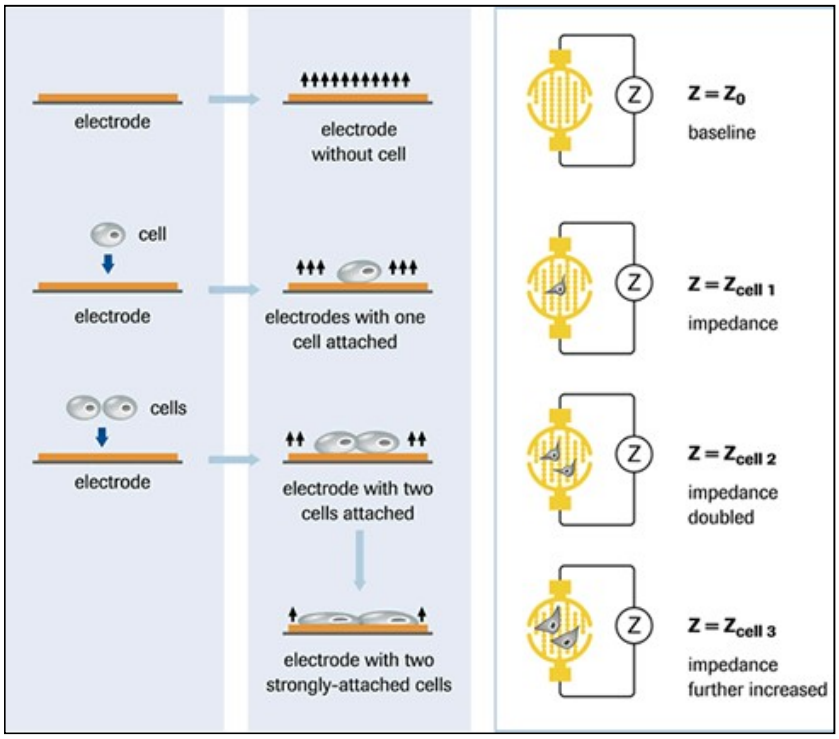


Fig. 15 Measurement of impedance changes
Changes in the impedance result from the interactions of the cells with the microelectrodes, which are located at the bottom of each well of an E-plate. The impedance (Z), i.e. resistance of a circuit to alternating current, is zero, if no cells are present in the well or if they are not well attached to the bottom. The impedance (Z) will rise in the presence of cells and will even become larger, when more and more cells attach to the well. Cell adhesion also has an influence on the impedance. Strongly attached cells increase the impedance even further [95].

The relative change in the measured impedance is displayed as cell index (CI) values. These values represent cell status and are dimensionless. A *CI* value of zero indicates that either the cells were not present or not strongly attached to the bottom of the well. Large *CI* values suggest that more cells have adhered on the microelectrodes. Hence, *CI* value allows a quantitative estimation of cells present in one well. Changes in the *CI* value point to changes in cell adhesion, cell viability or cell morphology [95].

All the measured values are transferred to the laptop, where special software converts the measured impedance changes into *CI* values. These *CI* values are depicted in real-time. Also, the system can be used for many experiments in parallel, where read-out of each single well is possible, as each of them are equipped with electrodes [95].

The xCELLigence™ system is a newly developed tool from Roche using the technique of cellular impedance measurement. The aim here was to acquire knowledge and experience as to how the system works and to establish normal standard methods like the titration of YFV-17D

viral stocks and to determine the serum neutralization titer against YFV. The established protocols for YFV-17D titration and neutralization on this new system are presented in the following sub-chapters.

2.7.1 Viral titration by xCELLigence™ system

The principle of this assay and the performance are similar to the viral titration by $TCID_{50}$/mL.

For this assay, 45µL of DMEM media was put in each well of a 96-well E-plate. The virus was serially diluted with 5µL pipetting from one well to the other. Each dilution was applied in quadruplicate, as only when using several repetitions, the method becomes more accurate. After serially diluting the virus directly in the wells, 150µL of Vero E6 cells at a density of $3.3x10^4$ cells/ mL in DMEM medium were added to each well. The infected Vero E6 cells were cultured at 37°C in a humidified environment with CO_2 for seven days. After seven days, the curves were interpreted and the $TCID_{50}$ value was calculated after the formulae of Reed and Muench the same way as mentioned in the sub-chapter 2.6.2.

2.7.2 Microneutralization on xCELLigence™ system

For the microneutralization assay a 96-well E-plate is used. The principles and the mechanisms of the xCELLigence™ system have been described earlier in this chapter. The principle of a neutralization assay has been described in chapter 2.6.4.

50µL of DMEM media was placed in each well and to increase the humidity inside the plate 100µL of PBS were put in between the wells. Two-fold serum dilutions ranging from 1:8 to 1:512 were prepared directly in quadruplicates in each well in a total volume of 25µL per well. Prior to diluting each serum sample was heat-inactivated. 25µL of Vero B4-17D virus, which has been diluted to 100 $TCID_{50}$/ 25µL beforehand, was added and the plate incubated for one hour at 37°C. In the meantime, PS-cells were diluted to a density of 10^5 cells/mL and after one hour, 100µL of the cell suspension added to the wells.

In addition, a negative serum from a person who has not been vaccinated against YFV, was tested under the same conditions. Furthermore, a cell and a virus control were employed. For the cell control 100µL of cells of the same dilution as for the serum tested were incubated with 75µL DMEM medium and for the virus control, 25µL virus, 100µL cells and 50µL DMEM media were incubated.

The cells were cultured in DMEM with 10% FCS, 1% Glutamine, Streptamycin and Penicillin in a humidified environment with CO_2 for seven days. After seven days, the curves were interpreted and antibody titer was determined.

2.8 Immunogenicity studies of YF vaccine co-administered with MenAfriVac in healthy infants in Ghana and Mali

This chapter gives a detailed description of the two studies, which were performed in healthy infants in Ghana and Mali. The aim of the studies was the analysis of immunogenicity of the YF vaccine in these infants when administered concomitantly with a new meningococcal A conjugate vaccine (PsA-TT, MenAfriVac).

The studies were designed and conducted in accordance with the Good Clinical Practice guidelines established by the International Conference on Harmonization, and with the Declaration of Helsinki, and approved by the competent ethics committees and regulatory authorities. Both studies were coordinated by MVP, a partnership between the World Health Organization (WHO) and PATH, aiming to develop an affordable, monovalent, group A meningococcal conjugate vaccine through a public–private partnership with the vaccine manufacturer Serum Institute of India, Ltd [77].

Study A

The first study (PsA-TT-004) was a phase 2, double-blind, randomized, controlled, dose-ranging study to evaluate the safety, immunogenicity, dose response, and schedule response of PsA-TT administered concomitantly with local EPI vaccines in healthy infants. The study was conducted in rural northern Ghana from November 2008 to May 2012 and the main study results are reported by Hodgson et al (unpublished data). A total of 1200 infants were randomized to receive primary vaccination into 6 study groups of 200 subjects each. Subjects' group allocation during the study is presented in Table 12. Subjects in all groups received EPI vaccines (measles and YF) at 9 months of age. The EPI vaccines were administered alone in groups 3 and 4, and concomitantly with a second dose of PsA-TT with different dosages in groups 1A (10 µg), 1B (5 µg), and 1C (2.5 µg) and with a single dose of PsA-TT (10 µg) in group 2. Group 4 was the control group for this vaccine period (no blood draw was performed in group 3 at this time point).

Study B

The second study (PsA-TT-007) was a phase 3, double-blind, randomized controlled study to evaluate the immunogenicity and safety of different schedules and formulations of PsA-TT administered concomitantly with local EPI vaccines in healthy infants and toddlers. The study was conducted in urban Mali from March 2012 to September 2013, and the main study results are reported by Hodgson et al (unpublished data). A total of 1500 infants were randomized to receive primary vaccination into 5 study groups of 300 subjects each. Subjects' group allocation during the study is presented in Table 8. Subjects in all groups received EPI vaccines (measles and YF) at 9 months of age. The EPI vaccines were administered alone in group 3, and concomitantly with PsA-TT vaccine with different dosages in groups 1A (10 µg), 1B (5 µg), 2A (10 µg), and 2B (5 µg). Group 3 was the control group for this vaccine period.

Table 12: Summary Description of Two Infant Studies and Demographics of Study Subjects at Yellow Fever Vaccination

Study ID	Study Site	No. Of Subjects Enrolled/No. Of Subjects by Study Arm	Study Group	Vaccines Administered by Study Arm				
				At Age 14-18 wk: DTwPHBVHib-OPV in all Study Groups	At Age 9-12 mo: Measles-YF in all Study Groups	At Age 12-18 mo: DTwPHBVHib in all Study Groups	At Age 15-18 mo: Measles/Rubella in all Study Groups	No. Of Subjects at YF Vaccination, Sex: F/M, Age, Median (Min-Max)
Study A: PsA-TT-004	Navrongo, Ghana	1200/200 per group	1A 1B 1C 2 3 4	PsA-TT 10µg PsA-TT 5µg PsA-TT 2.5µg	PsA-TT 10µg PsA-TT 5µg PsA-TT 2.5µg PsA-TT 10µg	... ... PsA-TT 10µg ...	NA	1153 573/580 9 months (8-13 months)
Study B: PsA-TT-007	Bamako, Mali	1500/300 per group	1A 1B 2A 2B 3	NA	PsA-TT 10µg PsA-TT 5µg PsA-TT 10µg PsA-TT 5µg ...	NA	PsA-TT 10µg PsA-TT 5µg	1500 725/775 9 months (9-13 months)

Note: DTwPHBVHib - OPV: Diphtheria, Tetanus, Whole-Cell Pertussis (DTwP) vaccines, Hepatitis B vaccine (HBV), *Haemophilusinfluenzae* type B (Hib) and Oral Polio Vaccine (OPV); PsA-TT: Meningococcal A conjugate vaccine.

2.8.1 Immunogenicity

Blood samples obtained before and 4 weeks after YF vaccination were tested for neutralizing antibodies against YF virus in the microneutralization assay using the YF-17D target virus strain produced at the RKI in a concentration of 100 $TCID_{50}$ (tissue culture infectious dose, 50%)/well [96]. Neutralization titers (NTs) were expressed as the reciprocal serum dilutions yielding ≥50% neutralization after 5 days, that is, blocking at least 1 of 2 duplicate infections. A detailed description of this method and its evaluation can be found in chapter 2.6.4.
Seroconversion was defined as an *NT* at least twice as high as that at baseline (≥2-fold rise) 28 days after immunization. Seroprotection was defined as an *NT* ≥1:8.

2.8.2 Statistical Analysis

The statistical analysis for study A and study B was carried out by Yuxiao Tang from the global health organization PATH (Program for Appropriate Technology in Health).

The neutralizing geometric mean titers (GMTs) between the vaccine groups at baseline and four weeks after vaccination were compared using analysis of variance (ANOVA) adjusted for baseline titers, age, and sex. Percentages of subjects with NTs ≥2-fold rise and with NTs ≥1:8, along with their exact binomial 95% confidence interval (CI), were calculated. The 95% CI for the difference in the proportions of subjects with these responses between the control group and a particular study vaccine group where subjects received PsA-TT was computed using the Miettinen–Nurminen method [97]. If the upper limit of the CI was <10%, the response in the study vaccine group was considered to be noninferior to that of the control group. Reverse cumulative distribution curves of YF *NTs* were generated at baseline prior to vaccination and 4 weeks after vaccination. All immunogenicity analyzes were conducted in the intention-to-treat population. Missing values were treated as missing at random. All tests were 2-sided with a significance level of .05. Data analysis was performed using SAS, version 9.1.3.

3 Results

Results of the three projects are presented in this chapter, which is organized in three sections. The first section starts with depicting results of the two immunogenicity studies of YF vaccine co-administered with MenAfriVac in healthy infants in Ghana and Mali. In order to further analyze these samples in regard to YFV IgG and IgM antibody levels the second project aimed at establishing an indirect ELISA against YFV. The results of the procedure of establishing an ELISA against YFV are illustrated in the second section. The third section ends with presenting results of a microneutralization assay obtained with the xCELLigence™ system.

3.1 Immunogenicity studies of YF vaccine co-administered with MenAfriVac in healthy infants in Ghana and Mali

For study A (PsA-TT-004) a total of 1153 subjects (96% of the 1200 subjects enrolled at age 14 weeks) received YF vaccination at a median age of 9 months with a sex ratio (F/M) of 0.99. The immune response to yellow fever vaccine was assessed in all study subjects with sufficient volumes of sera.

In study B (PsA-TT-007) all 1500 subjects enrolled received YF vaccination at a median age of 9 months with a sex ratio (F/M) of 0.94. The immune response to yellow fever vaccine was assessed in a random subsample of 300 subjects with equal distribution in all study groups (60 subjects per group).

In order to evaluate immunogenicity of the YF vaccine co-administered with the new meningococcal A vaccine, MenAfriVac (PsA-TT), serum samples taken before as well as 28–35 days after vaccination were analyzed in regard to neutralizing antibodies against YFV.

3.1.1 Study A – PsA-TT 004

Distribution of YF *NT* values at baseline prior to vaccination according to study groups are shown in Fig. 16. As expected, titer values amongst vaccinees in the different groups lay between 1:2 to 1:6. Only a small number of vaccinees had titer values in the medium range from 1:8 to 1:31. One vaccinee even had a titer value of 1:512.

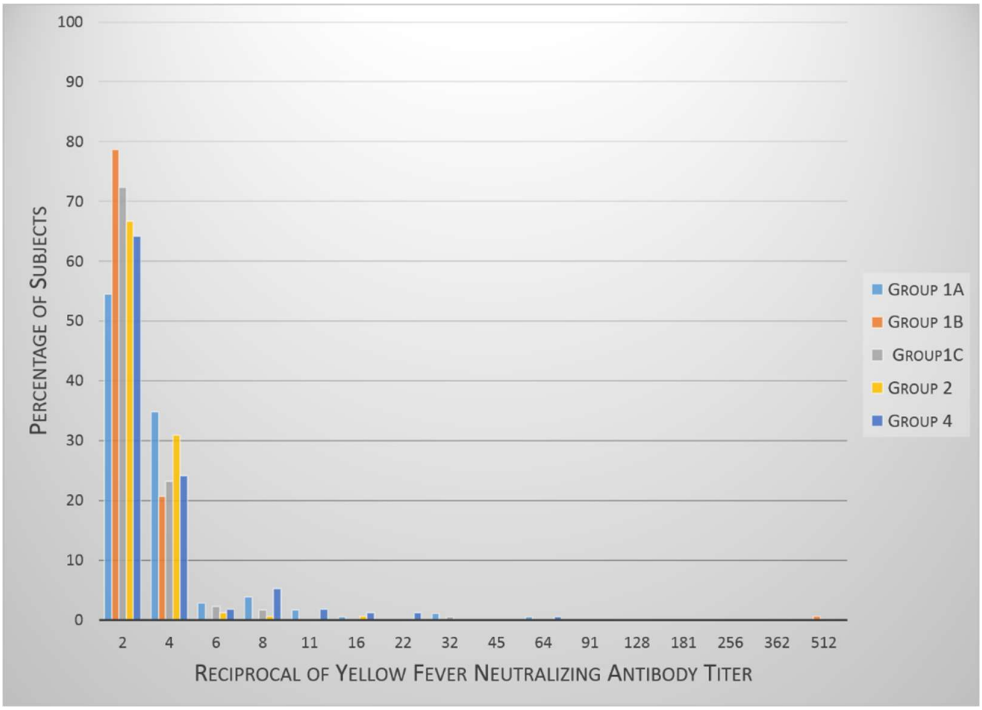


Fig. 16 Distribution Bars for Yellow Fever Neutralizing Antibody Titers prior to vaccination at 9 months of age and according to the vaccine group

The percentage of subjects with YF titers ≤1:8 was 4.5% (38/851), ranging from 0.6% (95% CI, 0.07%-3.4%) to 10% (95% CI, 5.9%-15.5%) in the vaccine groups. The geometric mean titers (GMTs) of YF titers ranged from 2.4 (95% CI, 2.2-2.6) to 3.0 (95% CI, 2.2-2.6) in the vaccine groups.

Four weeks after vaccination the distribution of titer values amongst vaccinees looked different, as shown in Fig. 17. The majority of vaccinees showed titer values that ranged from 1:8 to 1:32 and only a small number of vaccinees had titer values that lay in the high range from 1:45 to 1:512. Interestingly, almost the same number of vaccinees had titer values below the cut-off for sero-positivity of 1:8 (Fig. 17).

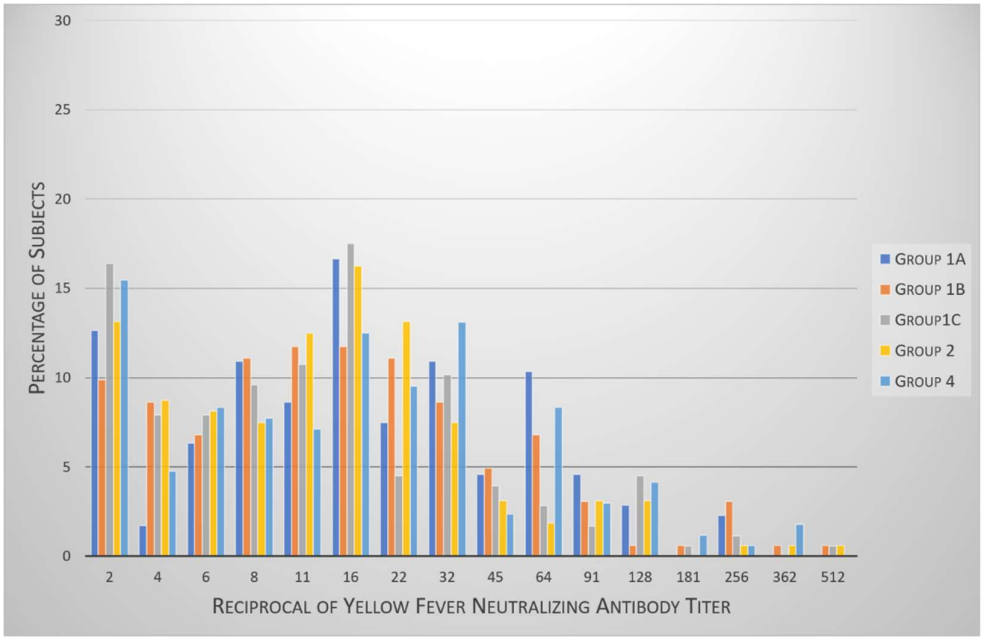


Fig. 17 Distribution Bars for Yellow Fever Neutralizing Antibody Titers at four weeks after vaccination and according to the vaccine group

YF neutralizing GMTs in each group were similar in all groups, ranging from 12.1 to 16.6, which is shown in Fig. 18. The method for comparison of all groups was ANOVA. No statistical significant difference was found after adjusting for age, sex and baseline titer.

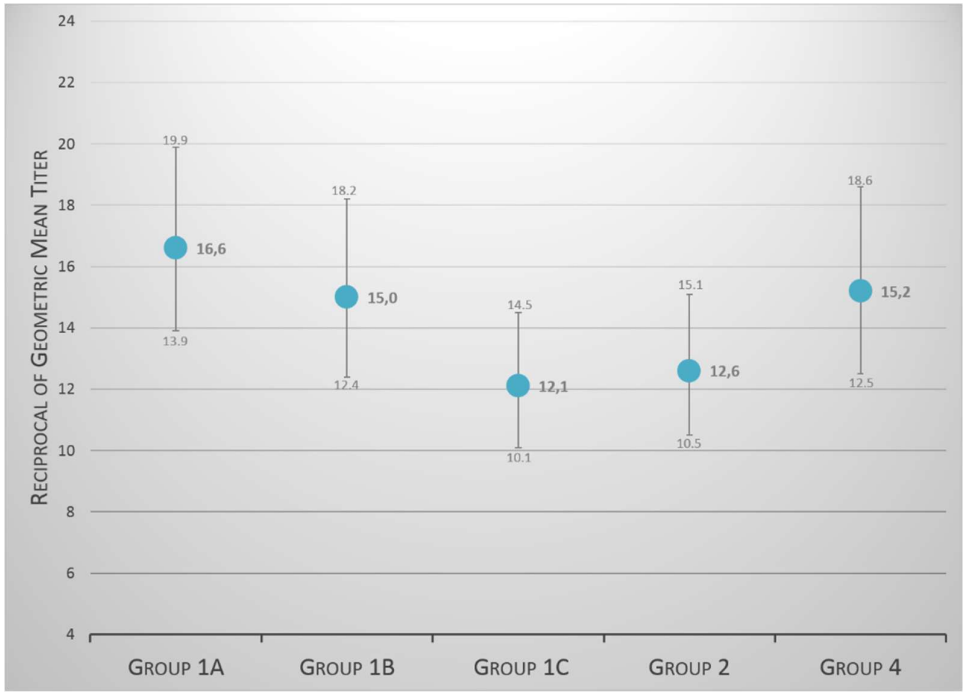


Fig. 18 GMT values with respective 95% CIs in each vaccine group four weeks after vaccination

The percentages of subjects with YF titers ≥1:8 four weeks after vaccination are shown in Fig. 19. These ranged from 67.8% to 79.3% and were lower than the anticipated response rate of 95% in all groups.

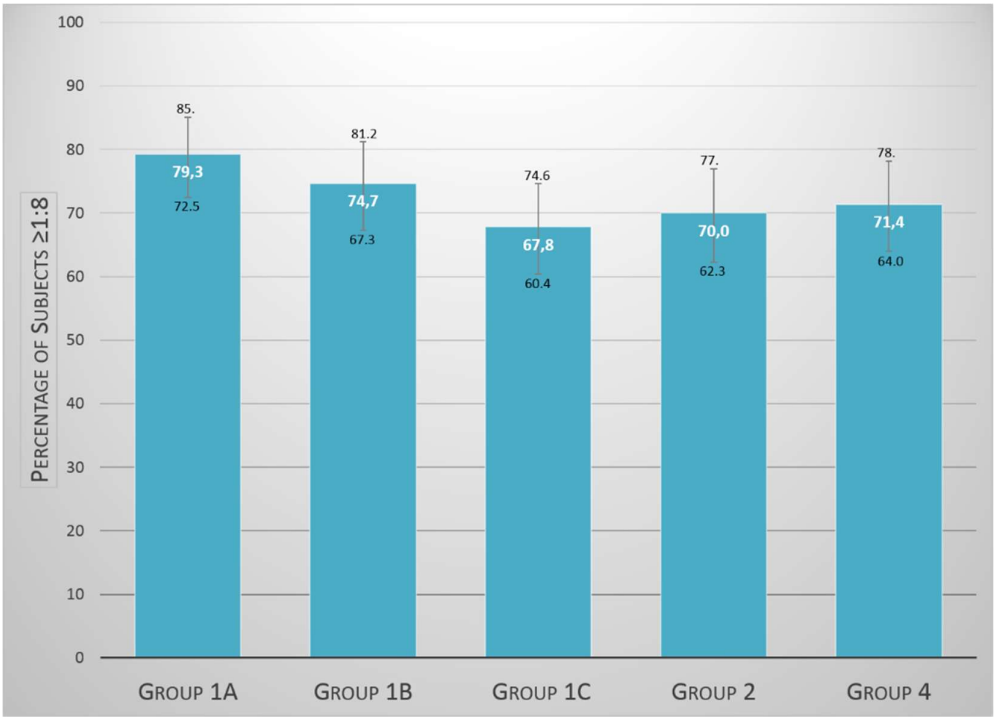


Fig. 19 Percentage of subjects with YF NTs above or equal to the cut-off for sero-positivity of 1:8 with respective 95% CIs in each vaccine group

Nevertheless, the non-inferiority of the immune response elicited by YF vaccine administered concomitantly with the second dose of PsA-TT vaccine at different dosages (10 µg and 5 µg) to that elicited by YF vaccine alone was demonstrated and is presented in the following figure (Fig. 20). As can be seen the upper limits of the 95% CI for the differences were <10% between group 4 and each of groups 1A and 1B. In contrast, the same non-inferiority was not confirmed when YF vaccine was administered concomitantly with the second dose of PsA-TT 2.5 µg vaccine or with the 1-dose 10 µg PsA-TT vaccine, with an upper limit of the CI of the difference of 13.3% between group 4 and group 1C and of 11.3% between group 4 and group 2, respectively.

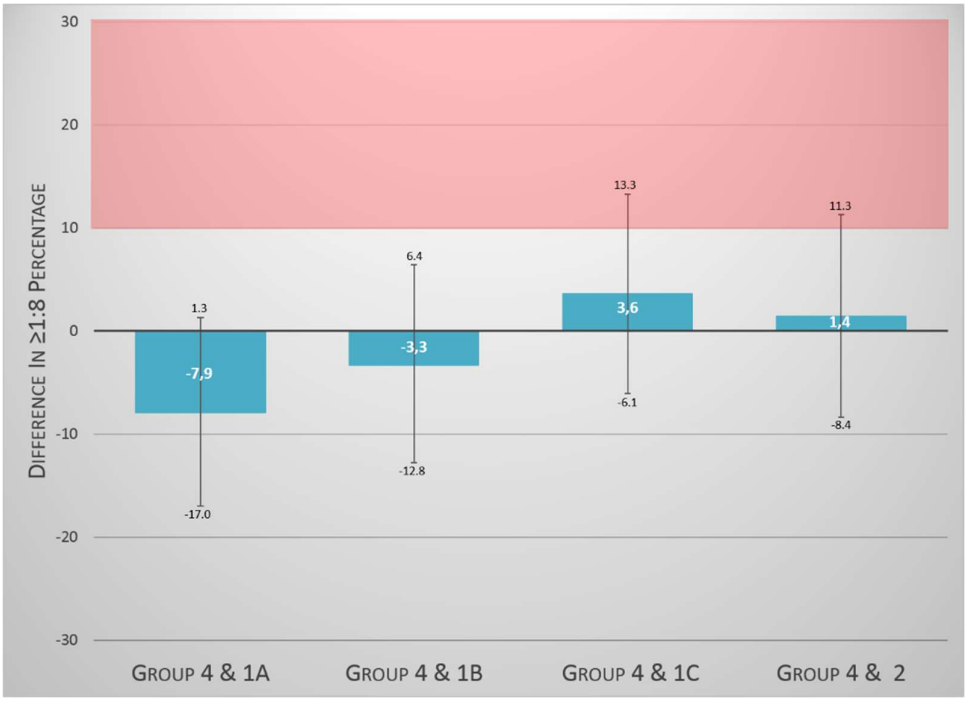


Fig. 20 Difference between the control group 4 and each of the vaccine groups where the percentage of subjects with YF NTs was ≥ 1:8

The red area marks the 10% boundary for non-inferiority. If the upper limit of the 95% CI of the difference between the control group 4 and each of the vaccine groups exceed this boundary, non-inferiority can not be confirmed. This is the case for group 1C and 2, where differences of the upper limits of the 95% CIs were above 10%. However, group 1A and 1B were non-inferior to the control group 4

In the following graph (Fig. 21) the percentages of subjects with a ≥ 2-fold response in YF titers with respect to baseline, i.e. those who have seroconverted are depicted. As can be seen these ranged from 64.8% to 71.0%

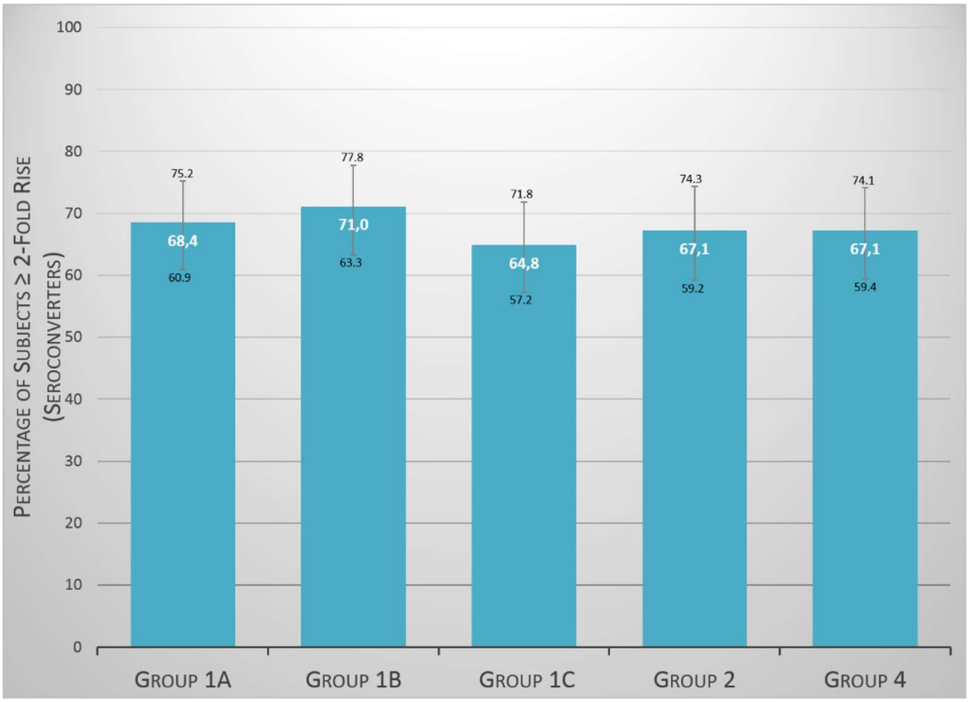


Fig. 21 Percentage of Seroconverters
Percentage of subjects, who had a 2-fold or higher response in YF titer values when compared to the baseline in each vaccine group with respective 95% CIs

The non-inferiority of the immune response elicited by YF vaccine administered concomitantly with the second dose of PsA-TT vaccine at different dosages (10 µg and 5 µg) to that elicited by EPI vaccines alone at 28 days after vaccine administration was demonstrated for this endpoint as well and can be seen in the following figure (Fig. 22).

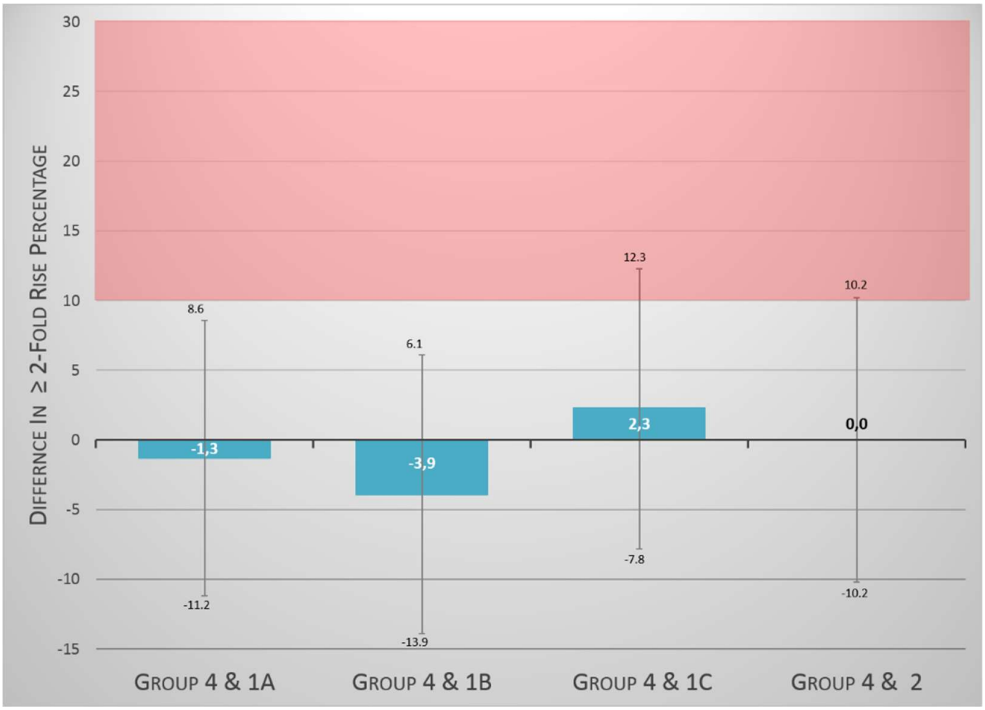


Fig. 22 Difference between the control group 4 and each of the vaccine groups where the percentage of subjects had ≥ 2-fold response in their YF NTs with respect to the baseline
The red area marks the 10% boundary for non-inferiority. If the upper limit of the 95% CI of the difference between the control group 4 and each of the vaccine groups exceed this boundary, non-inferioríty cannot be confirmed. This is the case for group 1C and 2, where differences of the upper limits of the 95% CIs were above 10%. However, group 1A and 1B were non-inferior to the control group 4

The upper limit of the 95% CI for the differences was <10% between group 4 and each of groups 1A and 1B. In contrast, the same non-inferiority was not confirmed when YF vaccine was administered concomitantly with the second dose of PsA-TT 2.5 µg vaccine or with the 1-dose 10 µg PsA-TT vaccine; that is, the upper limit of the 95% CI for the differences was 12.3% between group 4 and group 1C and 10.2% between group 4 and group 2.

The data was also analyzed in respect to gender differences four weeks post vaccination. Results are presented in Fig. 23 and Fig. 24. The analysis stratified by sex did not show any difference in the overall proportion of subjects with YF NTs ≥1:8. These were 72.6% [95% CI, 68.0%–76.9%] amongst girls and 72.7% [95% CI, 68.2%–76.8%] amongst boys. Also, the proportion of seroconverters (i.e. subjects with ≥ 2-fold YF neutralizing titer rises) did not show any difference between girls and boys. The percentage of seroconverters was 68.8% [95% CI, 64.1%–73.3%] amongst girls and 66.5% [95% CI, 61.8%–71.0%] amongst boys.

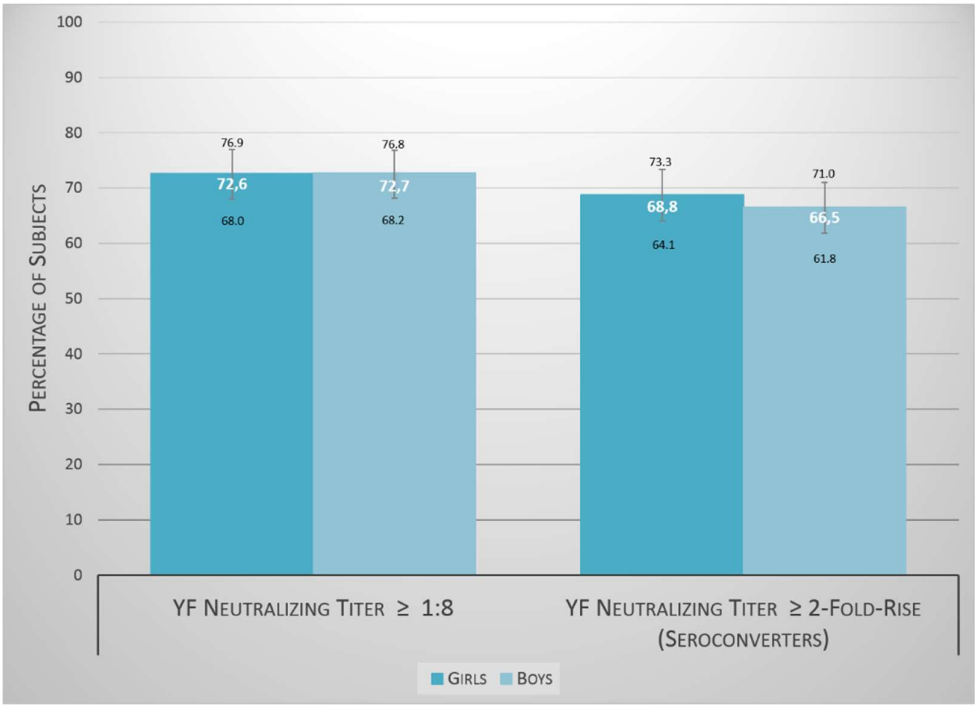


Fig. 23 Percentage of Girls and Boys with YF NTs above or equal to the cut-off for sero-positivity of 1:8 with respective 95% CIs four weeks after vaccination and Percentage of Girls and Boys, who had a 2-fold or higher response in YF titer values when compared to baseline with respective 95% CIs

The overall GMTs of YF fever neutralizing titers were 14.0 [95% CI, 12.4–15.7] amongst girls and 14.4 [95% CI, 12.8–16.2] amongst boys.

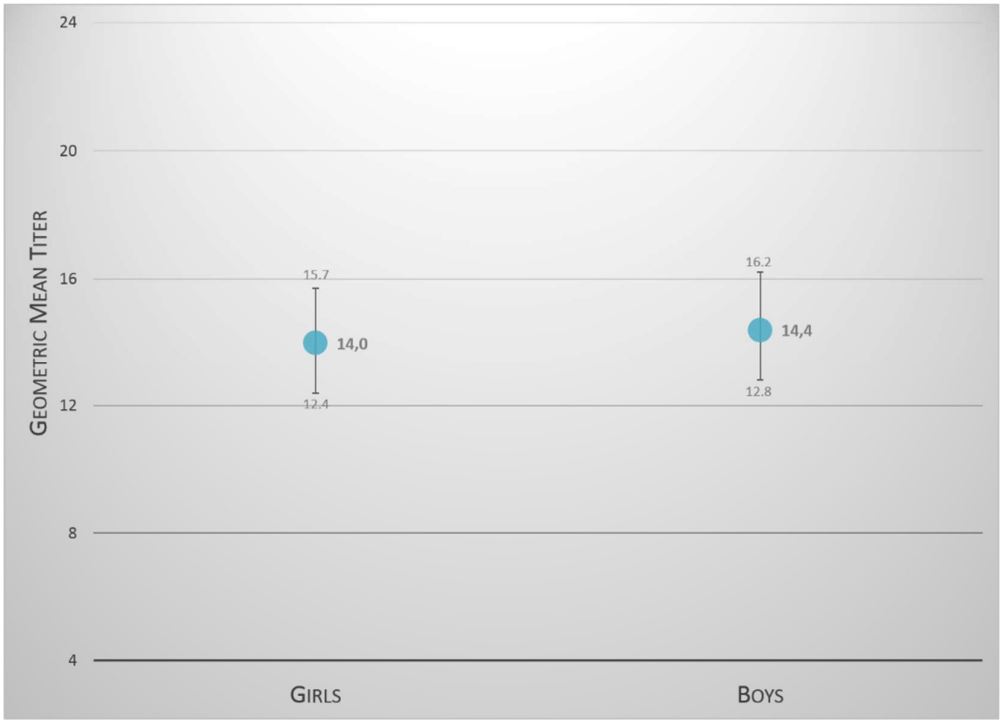


Fig. 24 Geometric Mean Titer Values with respective 95% CIs between girls and boys four weeks after vaccination

3.1.2 Study B– PsA-TT 007

Distribution bars for YF NTs at baseline prior to vaccination according to study groups, are shown in Fig. 25. As expected the YF *NT* at baseline prior to vaccination at 9 months of age was consistently low in all study groups. YF *NTs* amongst vaccinees lay between 1:2 and 1:6 in all study groups. Only a small number of vaccines showed titer values between 1:8 and 1:11.

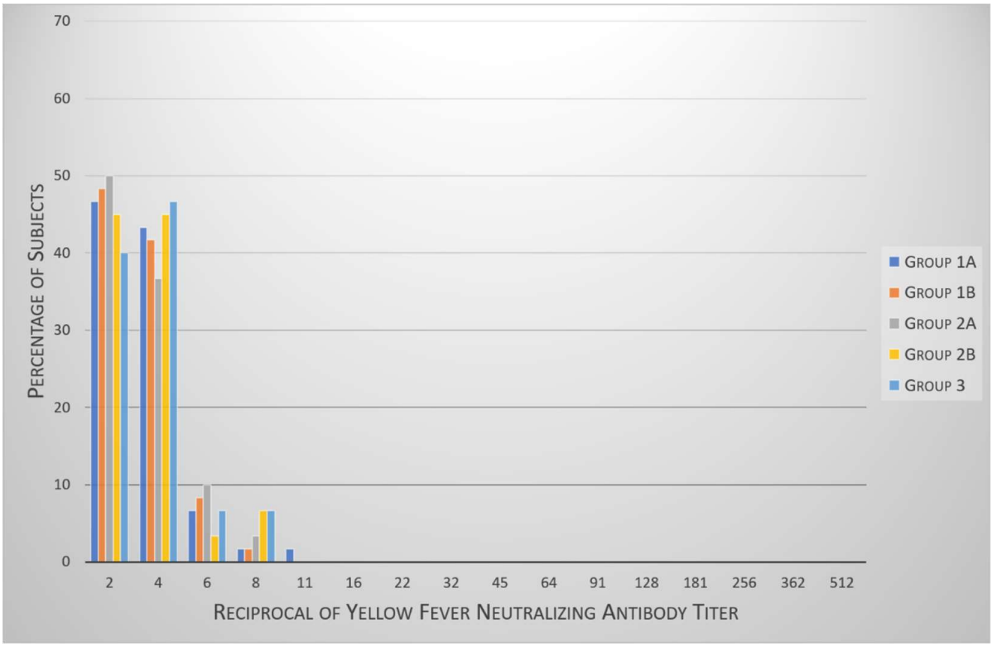


Fig. 25 Distribution Bars for Yellow Fever Neutralizing Antibody Titers prior to vaccination at 9 months of age and according to the vaccine group

The percentages of subjects with YF titers ≥1:8 at baseline prior to vaccination was 4.0% overall (12/300), ranging from 0.0% (95% CI, 0.0%–4.9%) to 6.7% (95% CI, 1.8%–16.2%) in the different study groups. The GMTs of YF titers were low as well, ranging from 3.0 (95% CI, 2.7 –3.3) in group 1B to 3.3 (95% CI, 2.9–3.7) in group 3.

The opposite could be seen four weeks after vaccination. Distribution bars of YF *NTs* four weeks after vaccination and according to the vaccine groups are shown in Fig. 26. As can be seen, the distribution of YF *NTs* was consistently similar in all study groups. The majority of vaccines showed titer values which were in the higher range from 1:22 to 1:64. Only a very small amount of vaccinees had YF *NTs* below the cut-off for sero-positivity of 1:8.

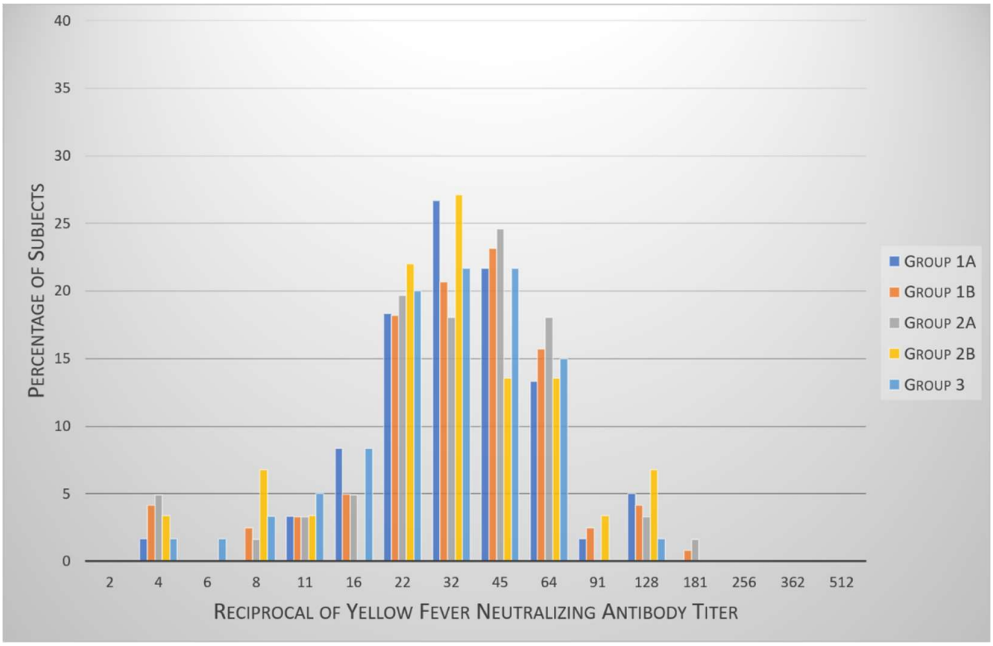


Fig. 26 Distribution Bars for Yellow Fever Neutralizing Antibody Titers at four weeks after vaccination and according to the vaccine groups

YF neutralizing GMTs four weeks after vaccination are presented in Fig. 27. When looking at these it is noticeable that, here too, values were similar in all groups ranging from 29.1 to 33.9, with no statistically significant difference when groups were compared using ANOVA after adjusting for age, sex, and baseline titer.

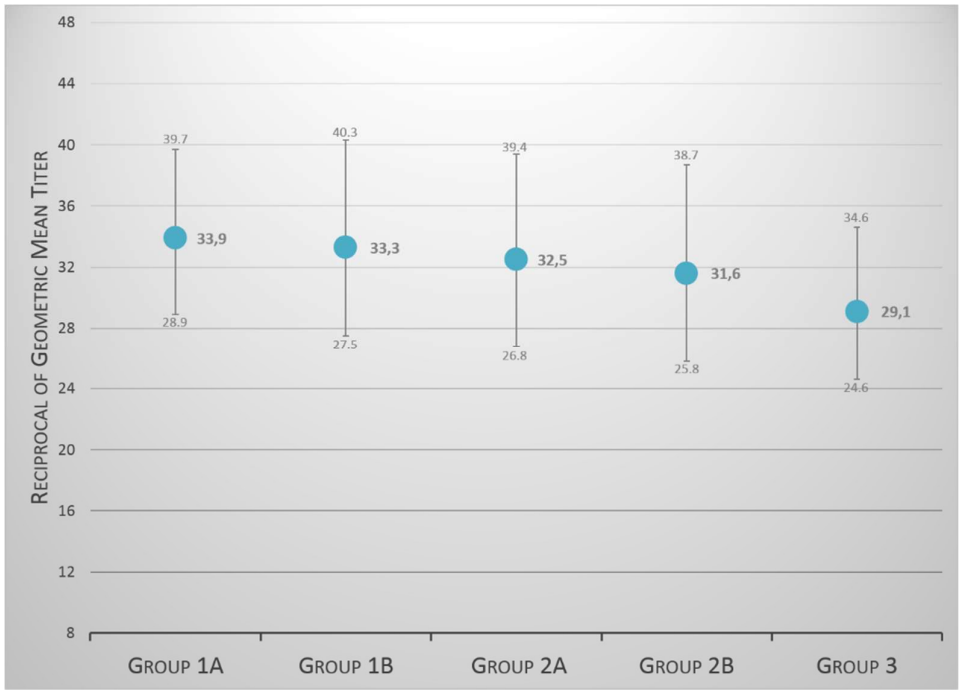


Fig. 27 Geometric Mean Titer Values with respective 95% CIs in each vaccine group four weeks after vaccination

The percentages of subjects with YF titers ≥1:8 four weeks after vaccination are presented in Fig. 28. These were higher than in study A and also above the anticipated response rate of 95% ranging from 95.1% to 98.3%. As can be seen the percentages were similar in all study groups.

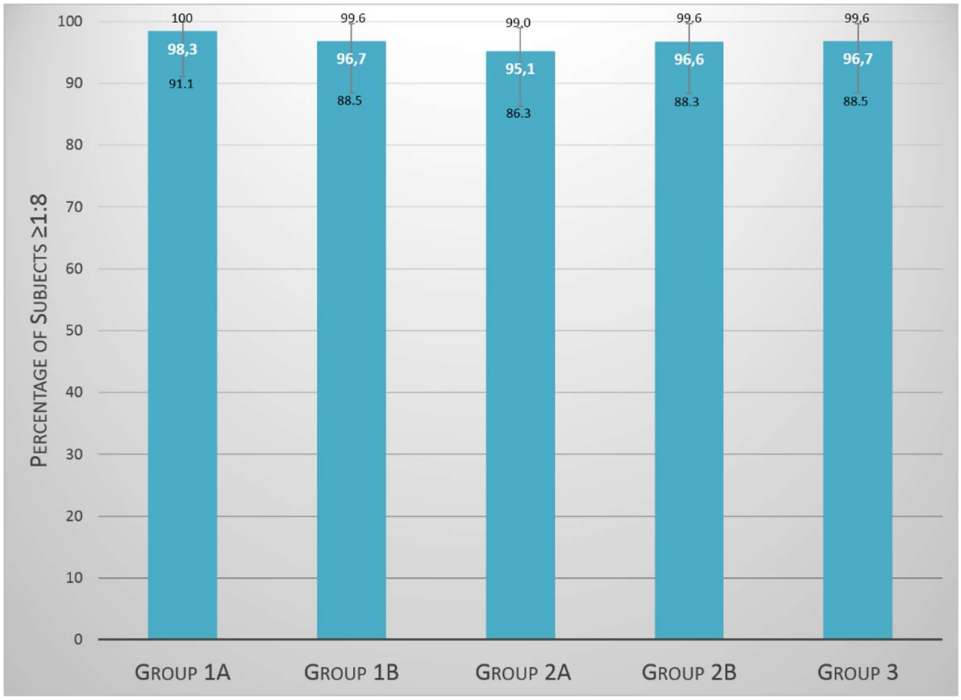


Fig. 28 Percentage of subjects with YF NTs above or equal to the cut-off for sero-positivity of 1:8 with respective 95% CIs in each vaccine group

The non-inferiority of the immune response elicited by YF vaccine administered concomitantly with PsA-TT at different dosages (10µg and 5µg) to that elicited by YF vaccine alone was demonstrated here as well (Fig. 29). That is, the upper limits of the 95% CI for the differences were <10% between group 3 and each of groups 1A, 1B, and 2B. However, Fig. 29 also shows that the same non-inferiority of group 2A (YF and PsA-TT 10 µg vaccines) to group 3 (YF vaccine alone) was not confirmed with respect to the same endpoint; that is, the upper limit of the 95% CI for the difference was ≥10% (10.7% between group 3 and group 2A).

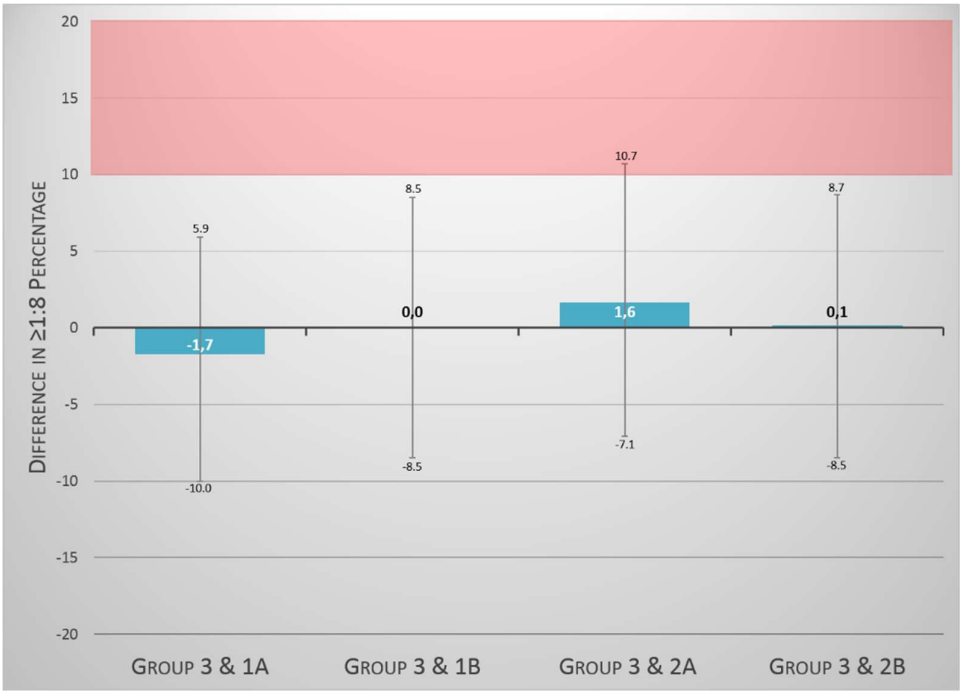


Fig. 29 Difference between the control group 3 and each of the vaccine groups where the percentage of subjects with YF NTs was ≥ 1:8

The red area marks the 10% boundary of non-inferiority. If the upper limit of the 95% CI of the difference between the control group 3 and each of the vaccine groups exceed this boundary, non-inferioríty can not be confirmed. This is the case for group 2A only, where the difference of the upper limit of the 95% CI was above 10%. However, group 1A,1B and 2B were non-inferior to the control group 3

The percentages of subjects with a ≥2-fold response in YF titer with respect to baseline, i.e. the percentage of subjects, who have seroconverted are shown in Fig. 30. These ranged from 89.8% to 98.3%.

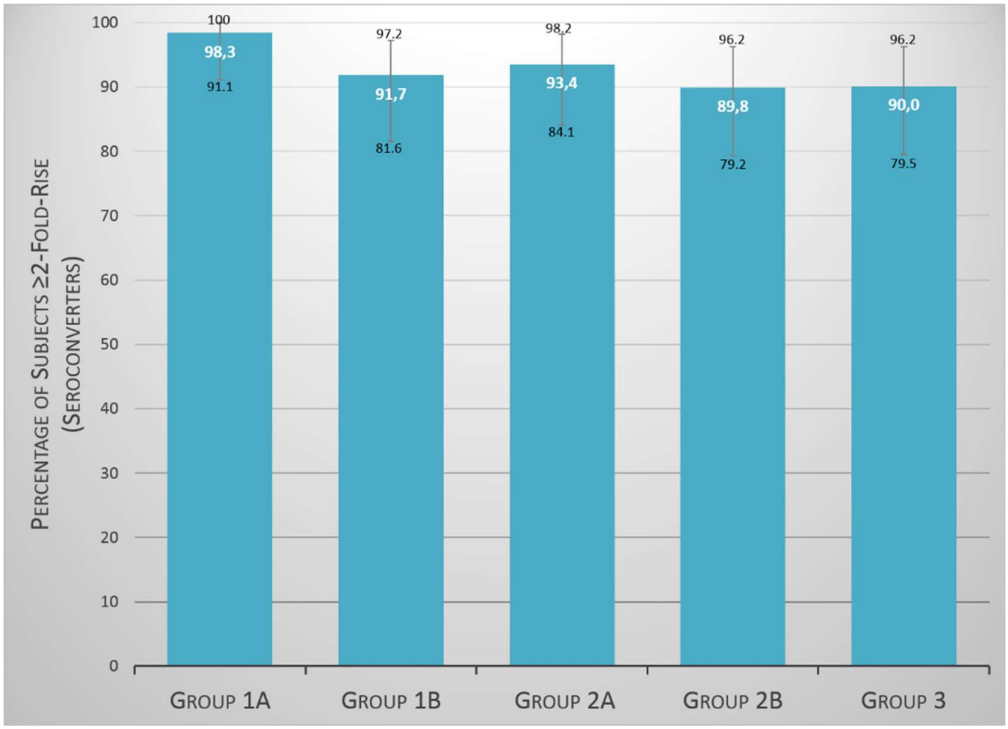


Fig. 30 Percentage of Seroconverters, i.e. percentage of subjects, who had a 2-fold or higher response in YF titer values when compared to baseline in each vaccine group with respective 95% CIs

The non-inferiority of the immune response elicited by YF vaccine administered concomitantly with the first dose of PsA-TT at different dosages (10 µg and 5 µg) to that elicited by YF vaccine alone was demonstrated for this endpoint as well and is presented in the next figure (Fig. 31).

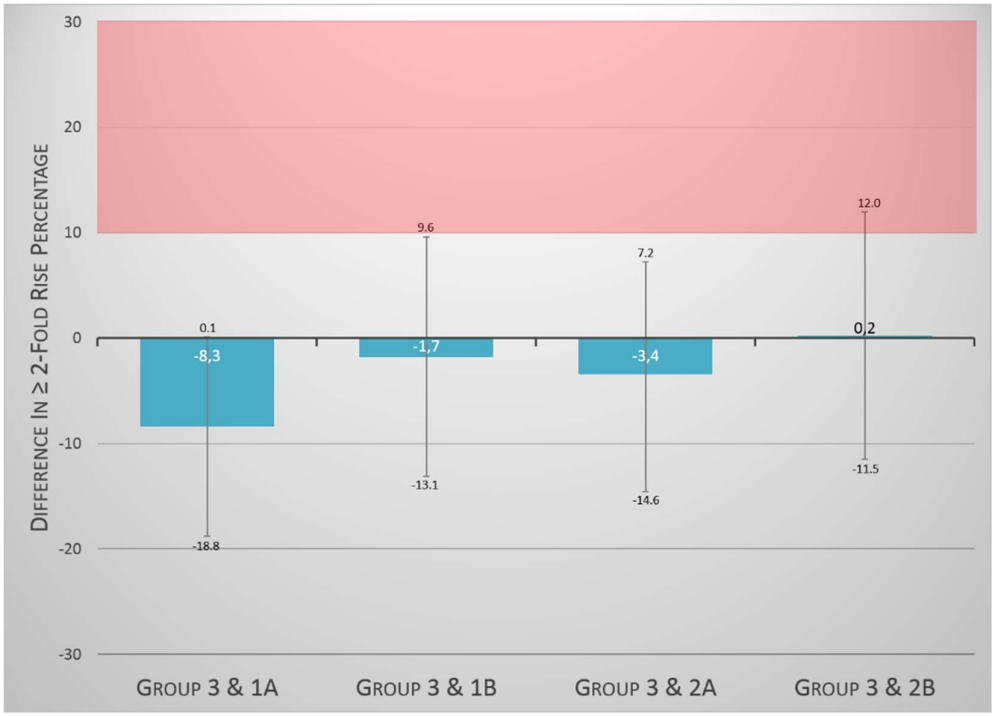


Fig. 31 Difference between the control group 3 and each of the vaccine groups where the percentage of subjects had a ≥ 2-fold response in their YF NTs with respect to the baseline The red area marks the 10% boundary of non-inferiority. If the upper limit of the 95% CI of the difference between the control group 3 and each of the vaccine groups exceed this boundary, non-inferioríty can not be confirmed. This is the case for group 2B, where the difference of the upper limit of the 95% CI was above 10%. However, group 1A, 1B and 2A were non-inferior to the control group 3

As shown, the upper limit of the 95% CI for the differences was <10% for each comparison of group 3 with groups 1A, 1B, and 2A. However, the same non-inferiority of group 2B (YF and PsA-TT 5-µg vaccines) to group 3 (YF vaccine alone) was not confirmed regarding the same endpoint; that is, the upper limit of the 95% CI for the difference was ≥10% (12.0% between group 3 and group 2B).

As in study A, gender differences four weeks post vaccination were analyzed here too. The results are depicted in Fig. 32. It was evident that analysis stratified by sex did not show any difference in the overall proportion of subjects with YF titers ≥1:8. These were 94.9% [95% CI, 89.7%–97.9%] and 98.2% [95% CI, 94.8%–99.6%] amongst girls and boys, respectively. Furthermore, the proportion of seroconverters, i.e. subjects with ≥ 2-fold YF *NT* rises did not show any difference between boys and girls with values of 91.2% [95% CI, 85.1%–95.4%] and 93.9% [95% CI, 89.1%–97.0%] amongst girls and boys, respectively.

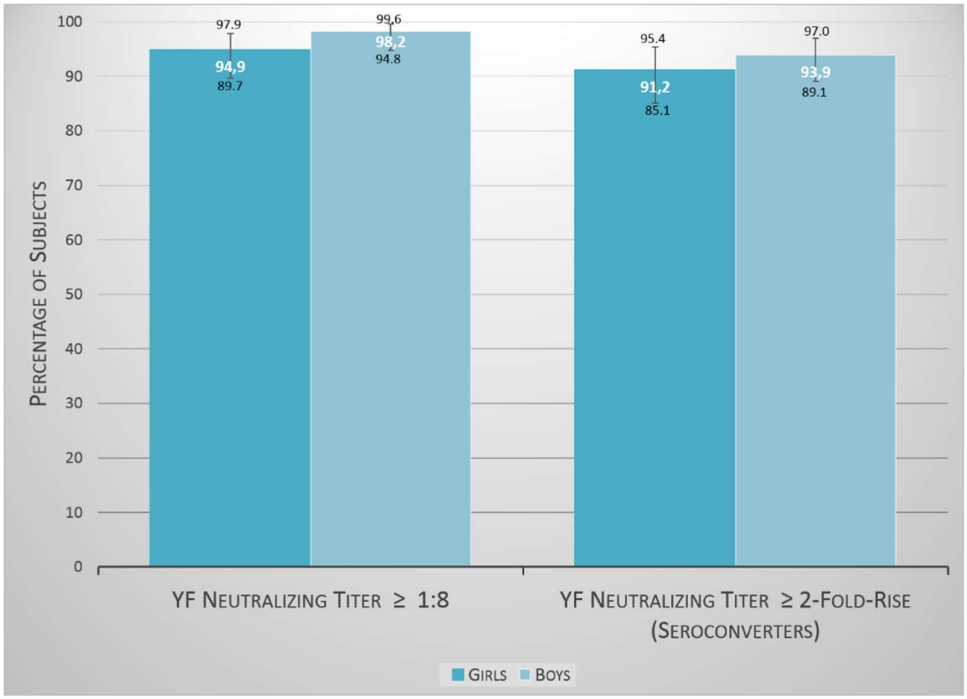


Fig. 32 Percentage of Girls and Boys with YF NTs above or equal to the cut-off for sero-positivity of 1:8 with respective 95% CIs four weeks after vaccination and Percentage of Girls and Boys, who had a 2-fold or higher response in YF titer values when compared to baseline with respective 95% CIs

The overall GMTs of YF fever NTs were 29.4 [95% CI, 26.1–33.1] and 34.4 [95% CI, 30.8–38.3] amongst girls and boys, respectively.

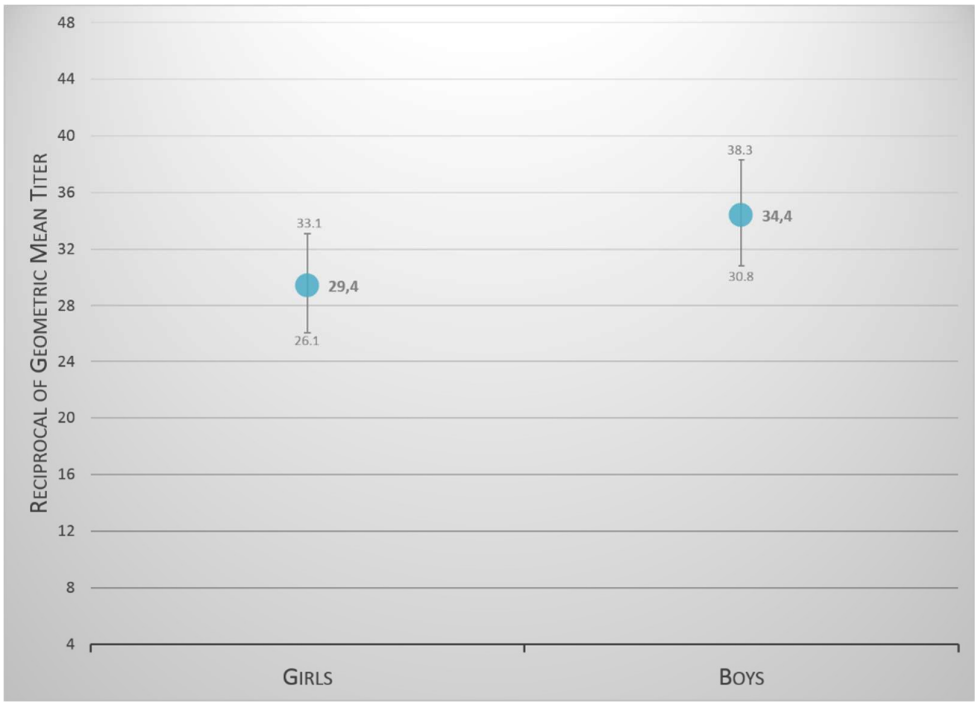


Fig. 33 Geometric Mean Titer Values with respective 95% CIs between girls and boys four weeks after vaccination

3.2 Establishment of an indirect enzyme-linked immunosorbent assay (ELISA) for the detection of YF IgG and IgM

This chapter is divided into three sections. The first two parts present results obtained during the process of establishing a YF IgG and YF IgM ELISA, respectively. The third section depicts results of the evaluation of the established YF IgG and IgM ELISA.

3.2.1 Establishment of an indirect IgG ELISA against YFV

The first experiment was to test different antigens diluted either with carbonate or citrate buffer in order to identify the specific combination giving the best result. All the antigens were tested in duplicates. The antigens recombinant E-III Domain and recombinant E-Protein were obtained for research purposes from the company NovaTec. Each of the antigens was challenged with a serum of a YF vaccinated person (+ serum) and with a serum of a non-vaccinated person (- serum). In Fig. 34 the mean absorbance values for each antigen are shown.

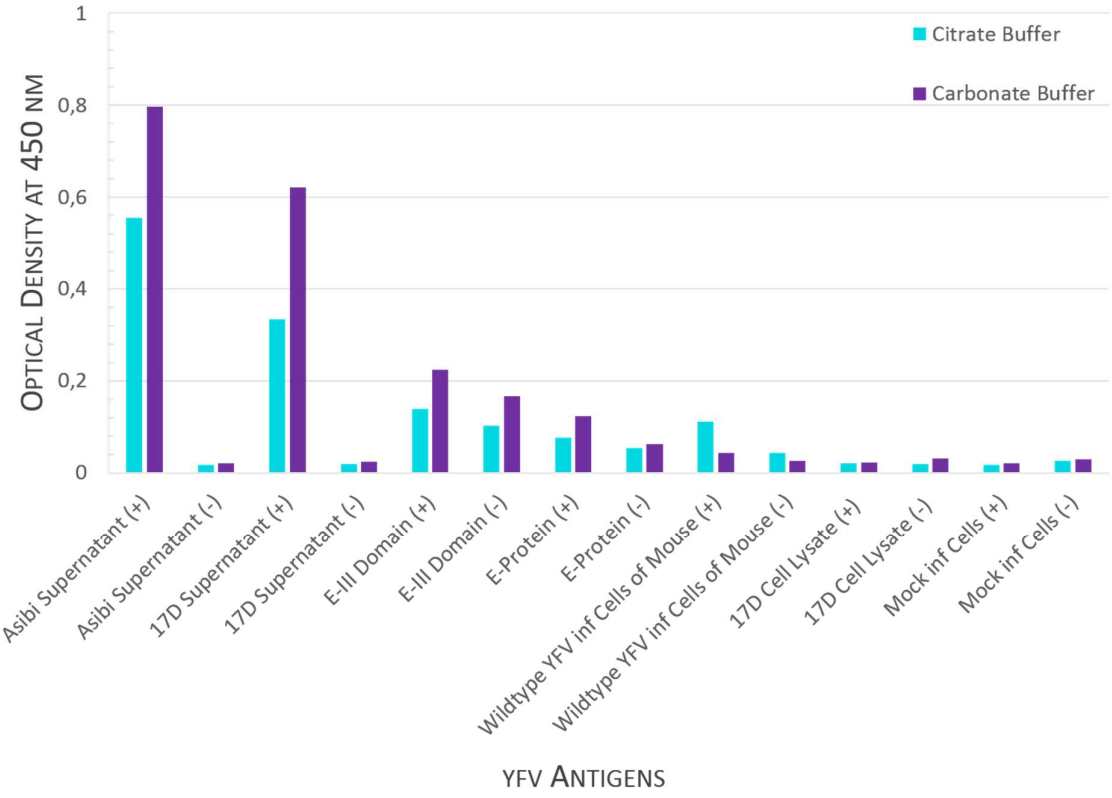


Fig. 34 Mean OD values of different YFV antigens diluted in either citrate (cyan) or carbonate buffer (purple). These antigens were challenged with a positive serum of a YFV vaccinee (+) and a negative serum of a non-vaccinee (-)

As can be seen, mock infected cells diluted in citrate and carbonate buffer and challenged with positive and negative serum showed an optical density below 0.1. The same is apparent for the 17D cell lysate. Generally, antigens diluted with carbonate buffer have higher absorbance values than the ones diluted with citrate buffer. The only exception here is wildtype YFV antigens of a mouse, where the values are higher when diluted with citrate buffer. Also, most of the antigens which have been challenged with the negative serum, have very low absorbance values (< 0.05) regardless what they have been diluted with. Exceptions here are the recombinant E-III Domain and the recombinant E Protein, where a clear statement cannot be made as the differences in the absorbance readings between positive and negative serum are so minor. The absorbance readings for the supernatant of 17D and Asibi infected were above 0.5 when diluted with carbonate buffer and challenged with the positive serum. Hence, these two antigens give the best results with carbonate buffer in this experiment.

The next step was to determine the best combination of an antigen concentration and a serum dilution for this assay. Therefore, wells of an ELISA plate were coated with different antigen dilutions and concentrations respectively. As the supernatant of Asibi infected cells gave good results in the previous experiment this antigen was applied here as well. Furthermore, supernatant of NS1 producing cells and the recombinant NS1 protein itself were used. The supernatant of Asibi infected cells and NS1 producing cells were used in dilutions starting from 1:10 to $1:10^4$. The NS1 protein was employed in concentrations ranging from 1µg to 0.06µg. Two-fold dilutions from 1:25 to 1:1600 of a positive serum were added to all these antigens. For all the experiments, the same positive serum was used. Several controls were added in this experiment as well. These were serum (only serum and detection antibody), antigen in a cocentration of 1µg per well (AG – only antigen and detection antibody), antibody (AB – only detection antibody) and substrate control (only substrate). Results in the form of mean absorbance values of the first two antigens – supernatant of Asibi infected cells and NS1 producing cells – are shown in the appendix (Fig. 61 and Fig. 62), results in the form of mean absorbance values of the third antigen, the recombinant NS1 protein are shown in following Fig. 35.

In summary to the first two antigens – results of the first antigen, the supernatant of Asibi infected cells, have shown that all the antigen dilutions of $1:10^2$ to $1:10^4$ have absorbance readings around the value of the serum control. Therefore, all the signals of this antigen with the respective serum dilutions are considered as background signal. Only the antigen dilution of 1:10 in combination with the lowest serum dilution of 1:25 gave a clear signal of 0.35. The exact same applies for the second antigen, supernatant of NS1 producing cells.

The last antigen tested was the recombinant protein NS1. Here again the scheme of the assay was the same as mentioned above, i.e. the antigen was tested in different concentrations in combination with different serum dilutions. The results are shown in the next figure. The AG, AB and substrate control gave the expected results and absorbance values were below 0.03. In contrast to the other experiments the signals of the serum control were rather low and lay in the range of 0.2 for the lowest dilution of 1:25 to 0.02 for the highest dilution of 1:1600. The absorbance values for the antigen concentration of 0.06µg ranged from 0.4 for the lowest dilution to 0.03 for the highest serum dilution. The rest of the antigen concentrations all provided OD values above 0.5, which decreased eventually with increasing serum dilution.

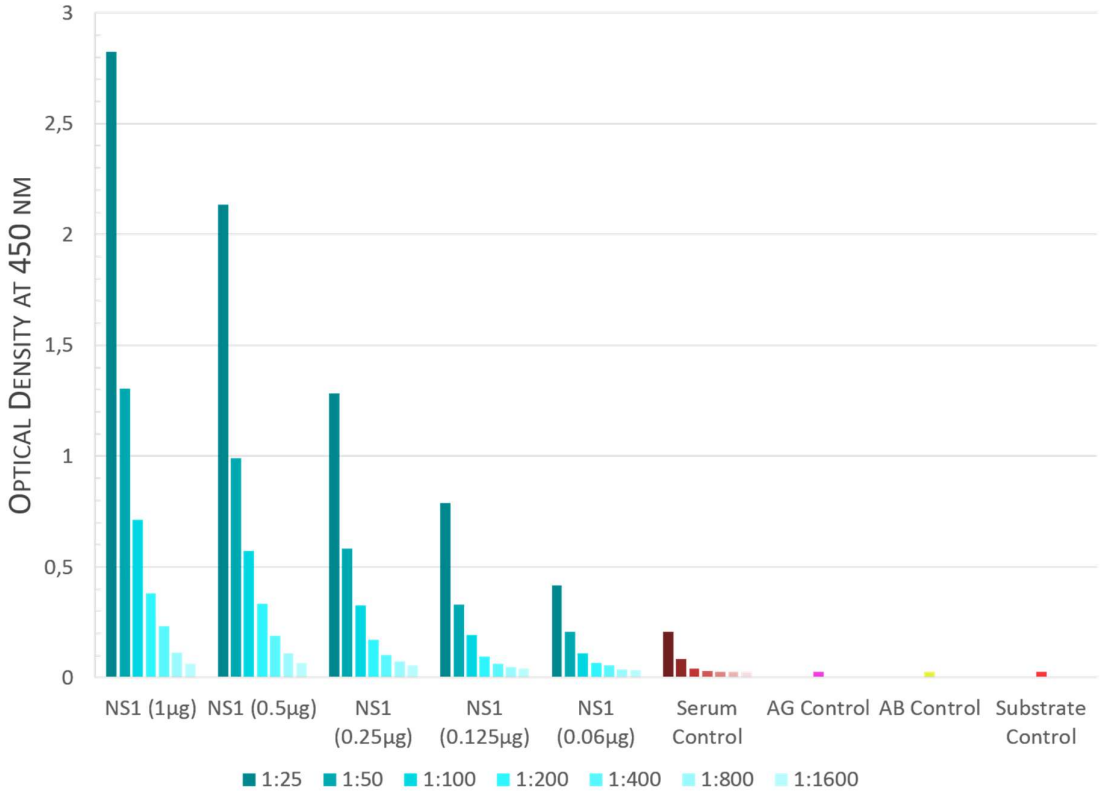


Fig. 35 OD values of different antigen concentrations of the NS1 protein starting from 1µg to 0.06µg for the detection of YF IgG antibodies.
To these dilutions different serum dilutions are added (graded colour bars) in order to identify the best combination of antigen concentration and serum dilution. The bars in brown present the serum control (only serum no antigen). The colour gradation also marks the increasing serum dilution. The pink bar depicts the antigen control (AG control), the light yellow bar the antibody control (AB control) and the red bar the substrate control

The highest OD value was provided by the antigen concentration of 1µg with a 1:25 serum dilution and lay at 2.83. This was followed by the 0.5µg antigen concentration with a 1:25 serum dilution and lay at 2.14. The rest of the antigen concentrations had absorbance readings

below 2.0 and varied according to serum dilution. Generally, the graded absorbance values of the different antigen concentrations together with the different serum dilutions were as expected. For all following assays, it was decided to work with a serum dilution of 1:50.

The next step was to test already characterized serum samples together with both the previously used antigens. The serum samples were already tested for IgG antibodies against YFV by an indirect immunofluorescence assay (IFA). This step was necessary to ensure the accuracy of this assay and to directly compare the antigens – supernatant Asibi and the NS1 protein. Therefore, wells of an ELISA plate were coated with these two at a dilution/concentration of 1:10 and 1µg respectively. To this 17 serum samples were added in duplicates at a 1:50 dilution (Fig. 36).

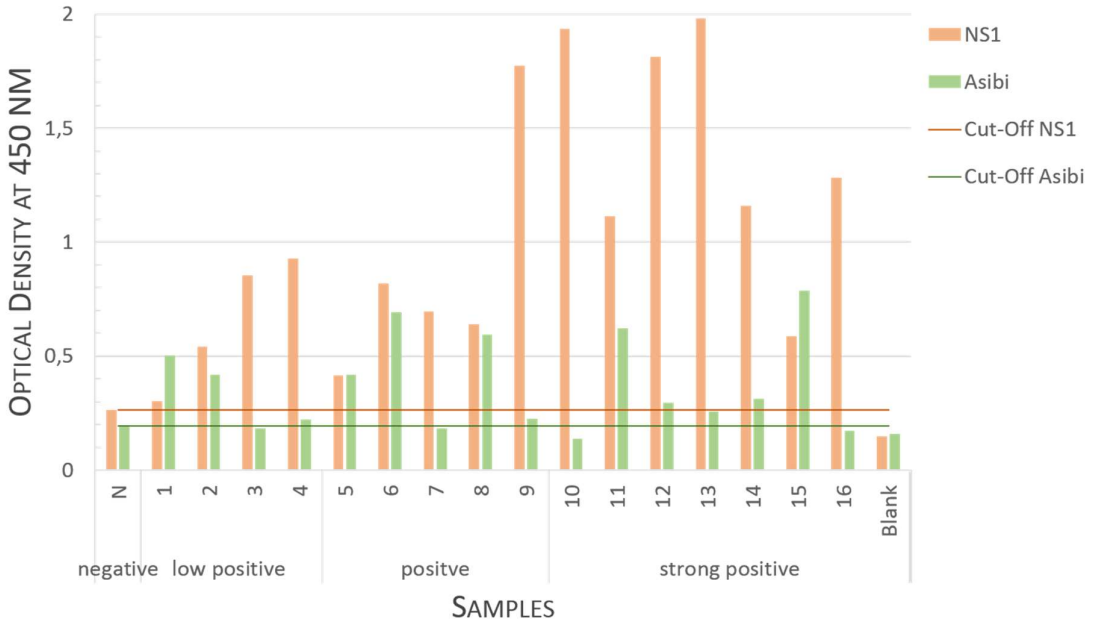


Fig. 36 Mean OD values of 17 serum samples for the detection of IgG antibodies.
These samples have been chaacterized by an IFA previously and are therefore grouped in negative, low positive, positive and strong positive. Furthermore, a blank sample is used as control. The dark orange line marks the cut-off for negativity in this assay for the antigen NS1 and the dark green line that for the antigen supernatant Asibi

As already mentioned these 17 samples were characterized by an IFA and hence were grouped in negative, low positive, positive and strong positive samples according to the IFA results.

Firstly, the negative serum N had an OD value of 0.36 with the antigen NS1 and 0.19 with Asibi as antigen. These values can be counted as negative and serve as cut-off values for negativity in this assay for the antigens NS1 and Asibi respectively. Generally, when looking at the results with NS1 as antigen a differentiation between low positive and positive samples cannot be

really made. However, strong positive samples by IFA are also strong in this assay giving OD values from 1.1 to 1.9. The only exception is the sample 15 with an absorbance reading of 0.5. Nevertheless, no false negative results could be seen with NS1 as antigen. In contrast, results with Asibi as antigen were different. Four samples 3, 7, 10 and 16, which were positive by IFA were all below the cut off value for negativity in this assay and hence are false negatives. Also, strong positive samples by IFA don't give the expected strong signals in this assay. Not one sample has an absorbance reading above 1.

As one of the aims of assay establishment is to reduce the quantity of specimen as much as possible, a last experiment was performed where two serum dilutions were tested. As antigen 1µg of NS1 was used. To this five serum samples in two different dilutions, namely 1:50 and 1:100, were added. These serum samples also have been characterized by IFA in negative, low positive and strong positive. The assay was performed the exact same way as before. The results in the form of mean absorbance values are shown in the next figure (Fig. 37).

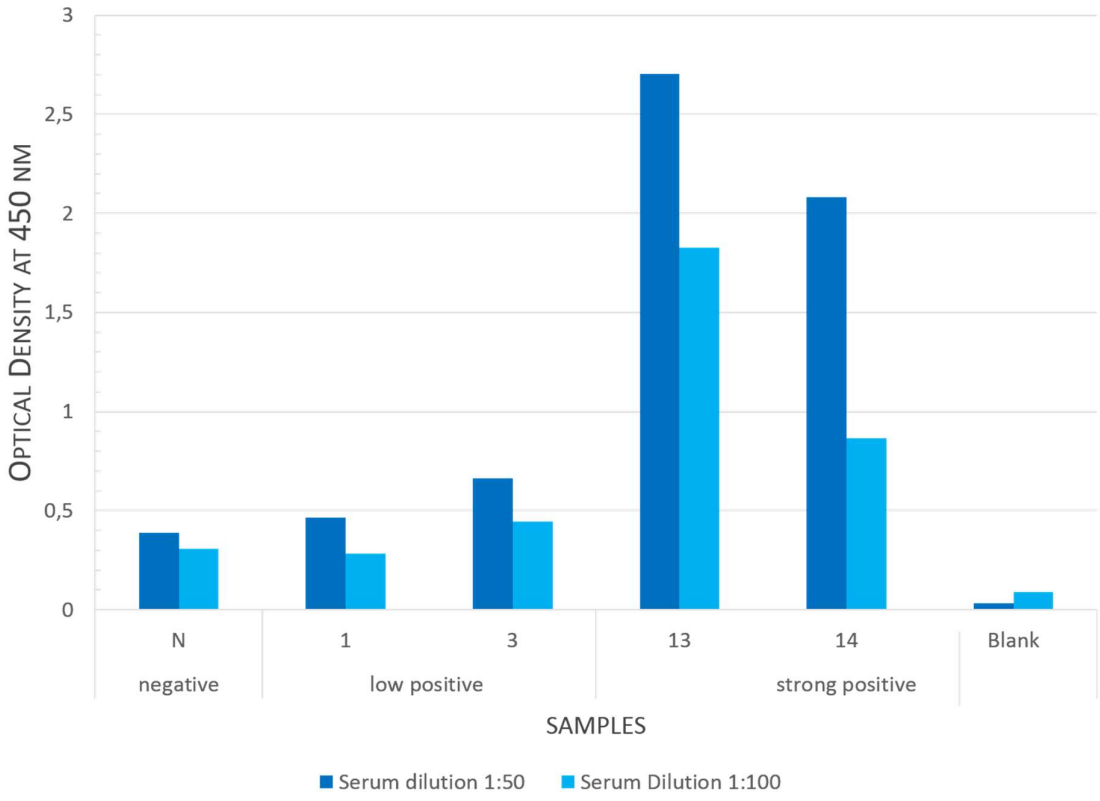


Fig. 37 The OD values of five different samples in two different dilutions - 1:50 (dark blue) and 1:100 (light blue). The samples have been characterized by an IFA previously and hence can be grouped in negative, low positive and strong positive samples. Additionally a blank sample has been included, which serves as control. As antigen 1µg of recombinant NS1 was used per well.

It can be seen that the 1:50 diluted samples give stronger signals than the 1:100 diluted samples. The difference between the 1:50 serum dilutions and the 1:100 serum dilutions is very big in the strong positive samples whereas for low positive samples this is not the case. The low positive sample 1 in the dilution 1:100 has an absorbance reading of 0,28, which is lower than the negative sample N (0.30) in the same dilution of 1:100 and hence would be considered as negative. In contrast, this same sample would be low positive in the 1:50 dilution as it has an OD value of 0.47, which lies slightly above the OD value of the negative sample N (0.39) in the same dilution. Also, the OD value for the blank in the 1:100 dilution is higher than for the 1:50 dilution, which could be due to background signal. Hence, for all further ELISAs 1µg NS1 was used as antigen and all the serum samples were diluted 1:50.

3.2.2 Establishment of an indirect IgM ELISA against YFV

For the establishment of an indirect IgM ELISA a similar approach is followed as with the establishment of the indirect IgG ELISA. Thus, the first step was to test the difference between carbonate and citrate buffer. As antigen 1 µg of NS1 per well was used in duplicates and challenged with two positive serum samples (pos serum 1 and pos serum 2). A negative serum sample and a blank sample served as control. Fig. 38 presents the mean absorbance values obtained here.

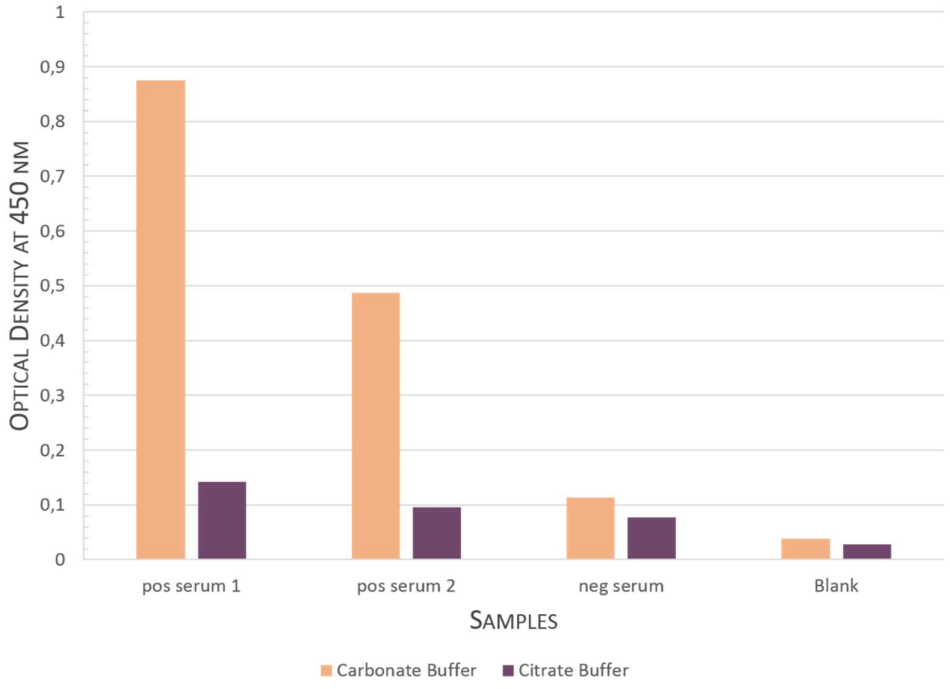


Fig. 38 Mean OD values of two positive serum samples, one negative serum sample and a blank sample as control diluted in either carbonate buffer (light orange) or citrate buffer (purple)

As shown above, the controls ‘neg serum’ and ‘Blank’ have very low OD values. Furthermore, the OD values, where antigens have been diluted with citrate buffer are distinctly lower than when diluted with carbonate buffer. This also applies to the other two positive samples. The clear difference between citrate and carbonate buffer is apparent. Hence, for all further experiments all antigens were diluted in carbonate buffer.

The next step was to test different antigens in duplicates. Each of the antigens was challenged with a serum of a YF vaccinated person (+ serum) and with a serum of a non-vaccinated person (- serum). Unfortunately, we did not have any of the antigens E-III Domain left anymore. However, we received three new antigens from the same company (NovaTec). These antigens were also parts of the E-III domain of the E-protein (E3 pp40, E3 pet44) as well as the full E protein (E-full 4T1)). The remaining antigens used here were the same as used in the IgG ELISA. In Fig. 39 mean of absorbance values of this experiment are shown.

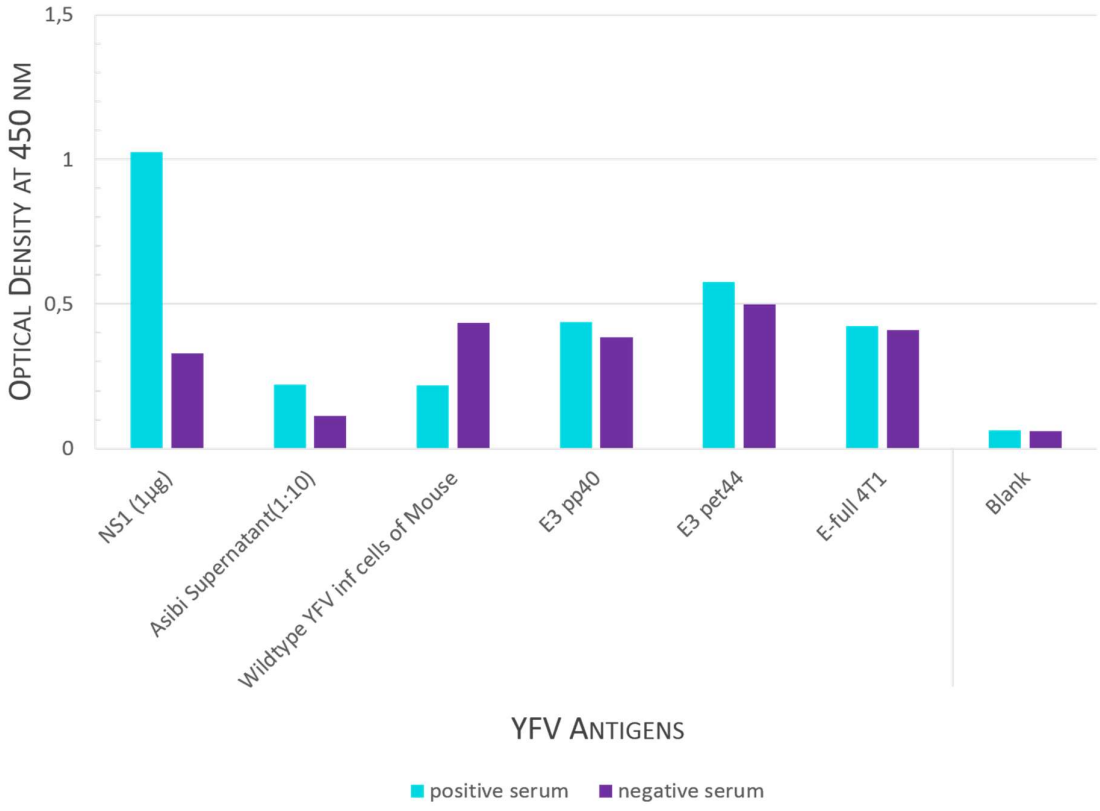


Fig. 39 Mean OD values of different YFV antigens
These antigens were challenged with a positive serum of a YFV vaccinee (cyan) and a negative serum of a non-vaccinee (purple). As control a blank sample was added

Firstly, the mean OD value for Blank is as expected and lies below 0.1 for the negative and the positive serum. Secondly, absorbance readings of the antigens E-full 4T1, E3 pet44 and E3

pp40 are relatively high with the negative serum (between 0.4 and 0.5) when compared to the positive serum indicating a high background signal. Moreover, these antigens have low OD values generally ranging from 0.4 to 0.6. Particularly noticeable is the absorbance reading of the antigen 'wildtype YFV infected cells of a mouse' with the negative serum, which is much higher than that with the positive serum. The OD value of this antigen with the negative serum is around 0.4 and 0.2 with the positive serum. Absorbance readings for the antigen 'Asibi Supernatant' are also rather low: 0.1 with the negative serum and 0.2 with the positive serum. In contrast, the antigen NS1 shows a good result with an OD value of around 1 for the positive serum and 0.3 for the negative serum. This also clearly demonstrates a difference between the positive and the negative serum.

In order to ensure that NS1 was the best antigen for this assay NS1 and the supernatant of Asibi infected cells were tested again with two positive serum samples – pos serum 1 and pos serum 3. Additionally, the µ-technology was also employed. The special feature of this technology is that wells are coated with anti-µ chain specific antibodies, which are specific to human IgM. Hence, any IgM antibodies in serum samples will be captured by the anti-µ chain antibodies. As detection antibody, a biotinylated monoclonal antibody (mAb 6330 biotinylated) against YFV-17D was used. Results of this experiment are presented in the figure below (Fig. 40).

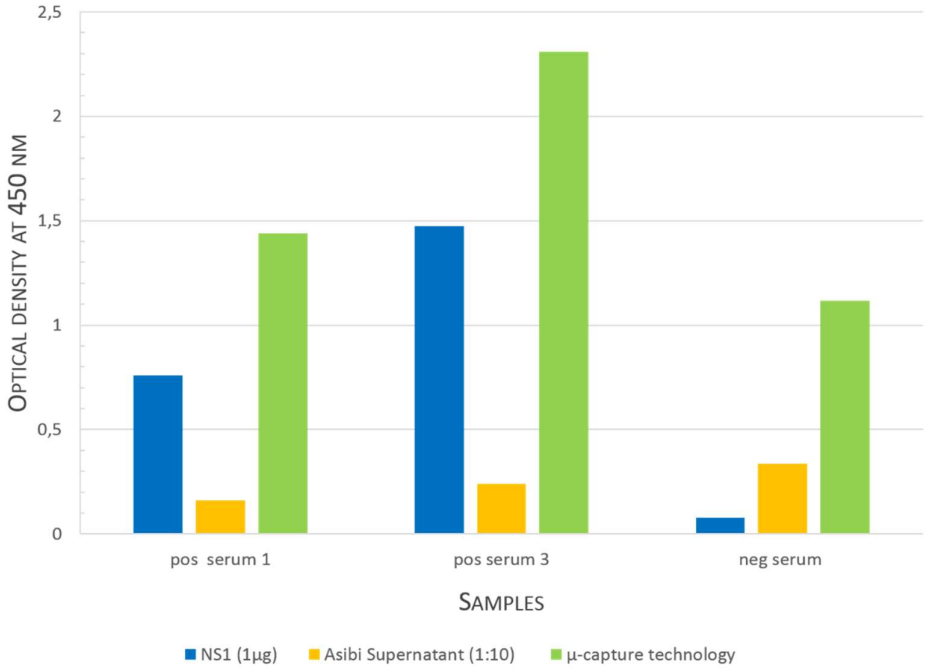


Fig. 40 Mean OD values of two different positive samples and one negative sample
For the detection of these two samples two different antigens were used – the NS1 protein (blue) and the supernatant of Asibi infected cells (yellow). Additionally, the µ-capture technology was tested (green).

As shown above, the negative serum has an OD value of 0.07 with NS1 as antigen, 0.3 with the supernatant of Asibi infected cells and 1.1 in the μ-capture ELISA, which is very high when compared to the other two antigens. Furthermore, when looking at the absorbance readings with the positive serum 1 and 3 it is noticeable that these are also rather high indicating high background noise. In contrast, the OD values for NS1 with the positive serum samples are 0.8 and 1.5 respectively and with the negative serum 0.07, which demonstrates a clear difference between positive and negative sample. The absorbance readings for the antigen 'Asibi Supernatant' is also rather low for the two positive samples (below 0.2). In fact, the OD value with the negative sample is slightly higher (0.3) than with the positive samples.

Next, the best combination of antigen concentration and serum dilution for this assay was determined. Therefore, wells of an ELISA plate were coated with two different antigen dilutions/concentrations ranging from 0.06μg to 1μg and $1:10^4$ to 1:10 respectively. As antigens, the NS1 protein and supernatant of NS1 producing cells were used. To these, two-fold dilutions from 1:25 to 1:1600 of a positive serum were added. For all experiments the same positive serum was used. Several controls were added in this experiment as well. These were serum (only serum and detection antibody), antigen (AG – only antigen and detection antibody), antibody (AB – only detection antibody) and substrate control (only substrate).

Results in the form of mean absorbance values of the first antigen – supernatant of NS1 producing cells – is shown in the appendix (Fig. 63), results in the form of mean absorbance values of the second antigen, the recombinant NS1 protein are shown in following Fig. 41.

In summary results of the first antigen, the supernatant of NS1 producing cells, have shown that the signals of the sample in all dilutions as well as antigen dilutions are very low. Hence, this antigen cannot be considered for further use in the assay.

The second antigen tested was the virus protein NS1. The results are shown in the next figure (Fig. 41). Once again AG, AB and substrate control gave the expected results and absorbance values were below 0.03. In contrast to the other experiment the signals of the serum control were rather low and lay in the range of 0.3 for the lowest dilution of 1:25 to 0.03 for the highest dilution of 1:1600. The absorbance values of the 1:25 serum dilution with the antigen concentration of 0.06μg and 0.125μg were rather high compared to the rest of the serum dilutions, which all lie in the same range. Hence, no clear gradation between these serum dilutions could be noticed.

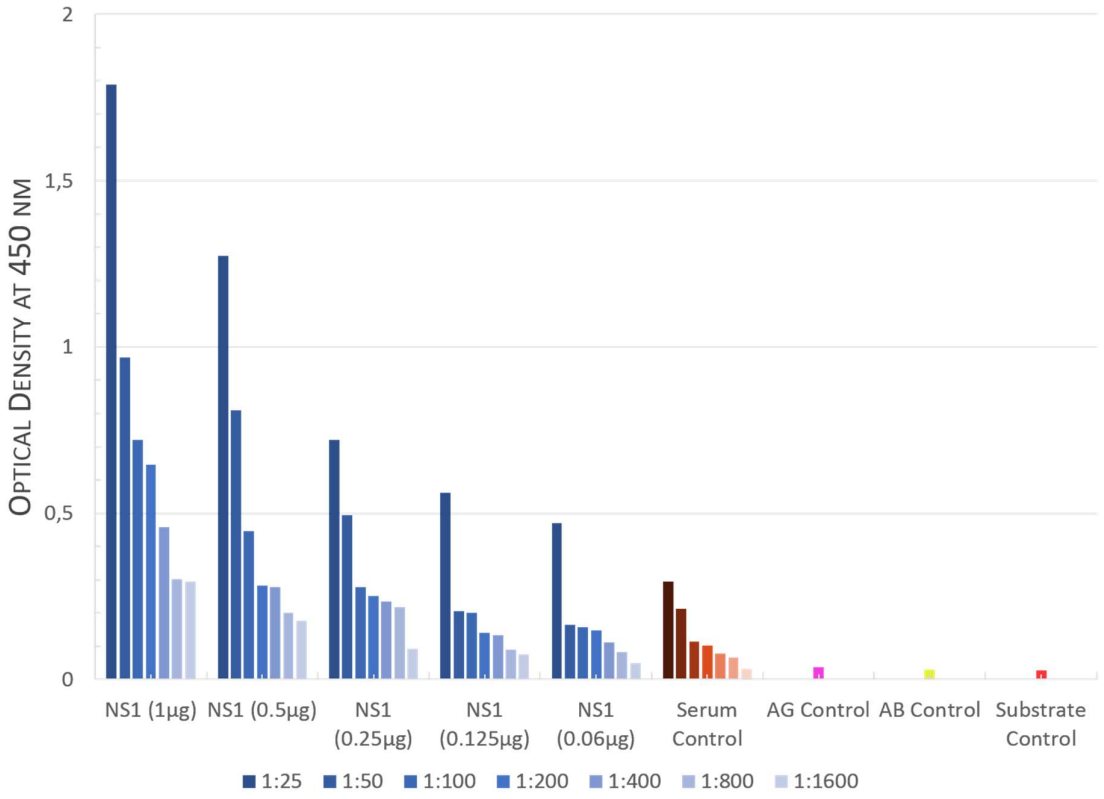


Fig. 41 Mean OD values of different antigen concentrations of the recombinant NS1 protein starting from 1µg to 0.06µg for the detection of YF IgM antibodies. To these dilutions different serum dilutions are added (graded colour bars) in order to identify the best combination of antigen concentration and serum dilution. The bars in brown present the serum control (only serum no antigen). The colour gradation also marks the increasing serum dilution. The pink bar depicts the antigen control (AG control – in a concentration of 1µg per well), the light yellow bar the antibody control (AB control) and the red bar the substrate control

This gets better with a higher antigen concentration of 0.25µg and 0.5µg. A clear gradation can be seen in the first two and three serum dilutions, respectively. Nevertheless, the best results were obtained by an antigen concentration of 1µg. The gradation of the signals is in accordance with the respective serum dilutions and hence can be clearly differentiated from each other. However, as the OD value of the serum dilution of 1:25 with an antigen concentration of 0.5µg is higher than the OD value of the 1:50 serum dilution with an antigen concentration of 1µg, it has to be decided with which antigen concentration and serum dilution to work with. As the quantity of sample material is always an issue, it was decided to work with an antigen concentration of 1µg of NS1 protein and a serum sample dilution of 1:50 for all further experiments.

Next, we tested serum samples, which were already tested for IgM antibodies against YFV by an indirect immunofluorescence assay (IFA). This step was necessary to ensure the accuracy of

this assay. Therefore, each well of an ELISA plate was coated with NS1 in a concentration of 1µg. To this 16 serum samples were added in duplicates at a 1:50 dilution. The results are shown in the next Figure (Fig. 42).

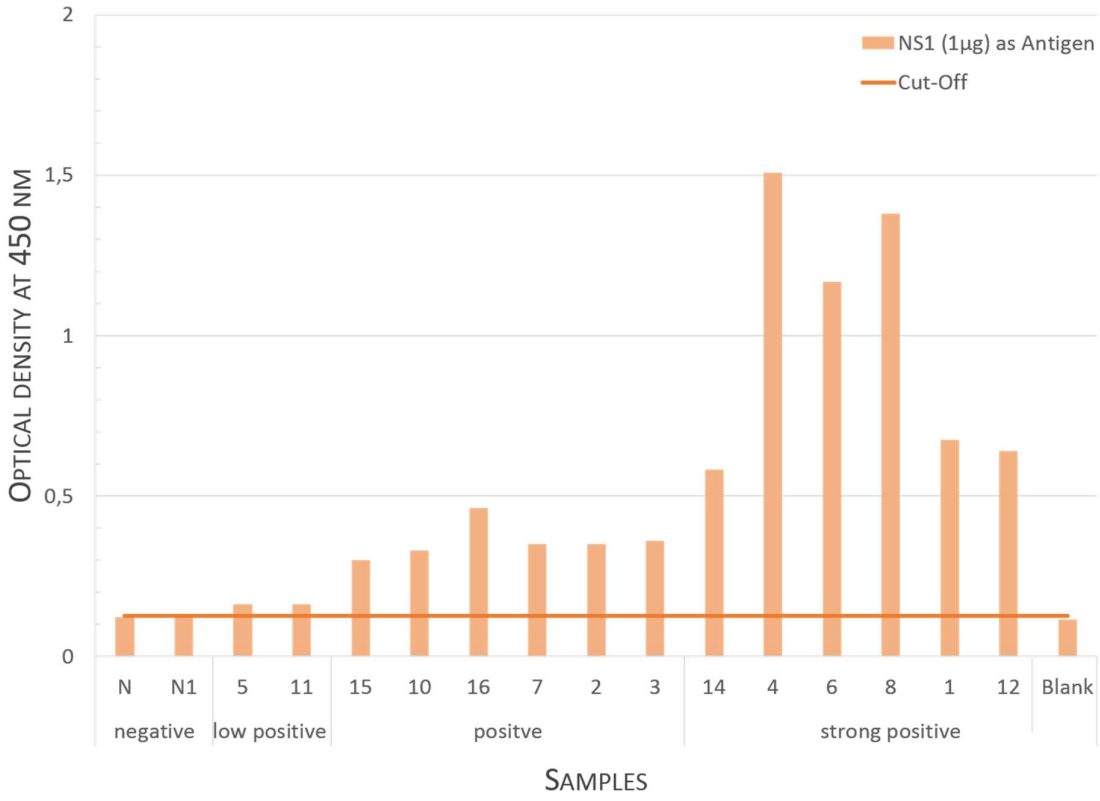


Fig. 42 Mean OD values of 17 serum samples for the detection of IgM antibodies.
These sample have been characterized by an IFA previously and therefore are grouped in negative, low positive, positive and strong positive. Furthermore, a blank sample has been added as control. The dark orange line marks the cut-off for negativity in this assay

As already mentioned these 17 samples were characterized by IFA and hence were grouped in negative, low positive, positive and strong positive samples.

First, as can be seen, the negative sera had OD values of 0.12 (N) and 0.126 (N1). These values can be counted as negative and serve as cut-off values for negativity in this assay. It is apparent from the results that differentiation between low positive, positive and strong positive samples can certainly be made. Furthermore, no false negative or false positive results can be seen here.

As one of the aim of assay establishment is to reduce the quantity of specimen as much as possible a last experiment was performed where two serum dilutions are tested again. As antigen 1µg of NS1 is used. To this four serum samples in two different dilutions, namely 1:50 and 1:100, were added. These serum samples have already been characterized by an IFA in

negative, low positive and strong positive. The assay was performed before. Results are shown in the following figure (Fig. 43).

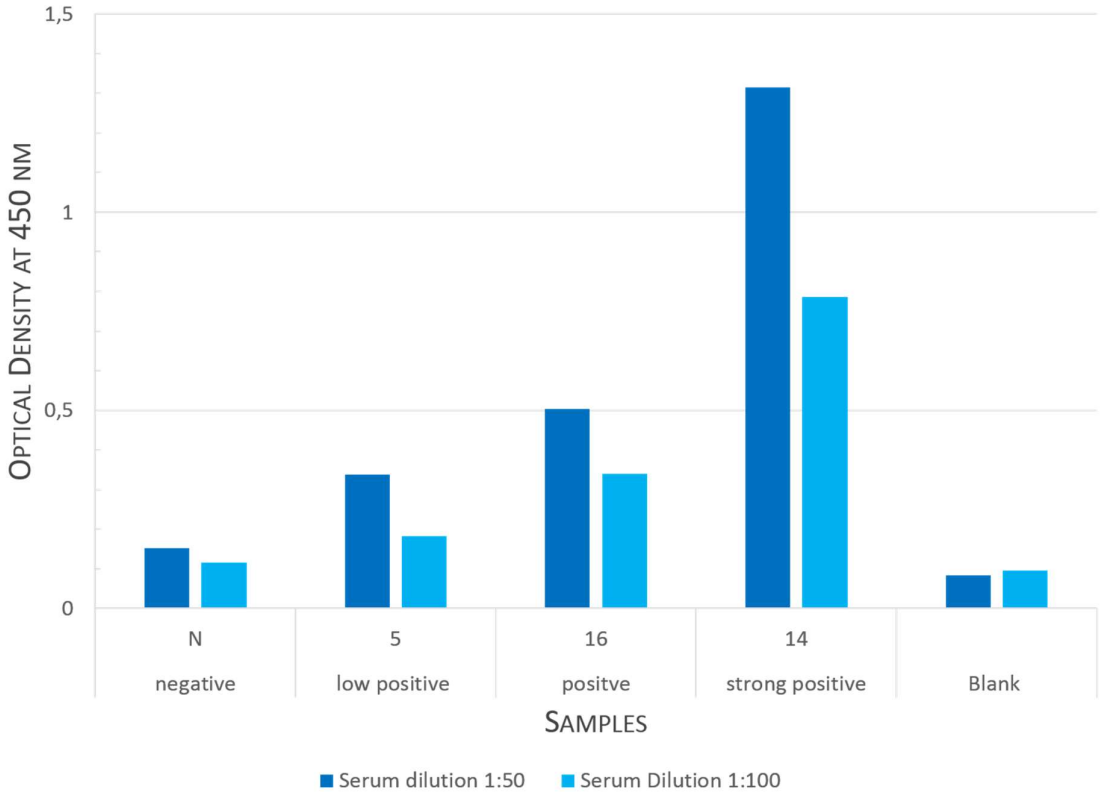


Fig. 43 Mean OD values of five different samples in two different dilutions - 1:50 (dark blue) and 1:100 (light blue) and a blank sample as control. The samples have been characterized by an IFA previously and hence can be grouped in negative, low positive, positive and strong positive samples.

At first glance, it can be seen that the 1:50 diluted samples give stronger signals than the 1:100 diluted samples. Here again, the OD value of the Blank sample in the 1:100 dilution is higher than for the 1:50 dilution, which could be due to background signal. As even for the strong positive sample the OD value for the 1:100 dilution lies below 1, it was decided to work with an antigen concentration of 1µg NS1 and a serum sample dilution of 1:50. After the evaluation of this experiment, the indirect NS1 based YF IgM ELISA against YFV was considered established.

3.2.3 Evaluation of the established indirect NS1 based YF IgG and IgM ELISA

In this chapter, the results of the evaluation of the newly established YF-NS1 ELISA are shown. The evaluation of the ELISA was accomplished by testing serum samples of study B (see chapter 2.8 Study B– PsA-TT 007) for YFV IgG and YFV IgM antibodies. As these samples

have been already tested and analyzed by a microneutralization assay they have known YFV *NT*. Hence, results of both assays can be compared to draw a conclusion on the performance of this assay.

A total of 190 YFV positive serum samples and 187 YFV negative serum samples of vaccinees have been analyzed by this indirect IgG and IgM ELISA. The negative serum samples gave *NT* values between >1:4 to 1:6 and hence were confirmed negative serum samples.

In order to discriminate between positive and negative samples, a cut-off value was calculated. The previously mentioned 187 negative serum samples were used for this and were tested by the IgG and IgM ELISA respectively.

In the following figure (Fig. 44 A, B) mean absorbance values of all the negative samples for the detection of IgG (Fig. 44 A) as well as IgM antibodies (Fig. 44 B) against YFV are presented.

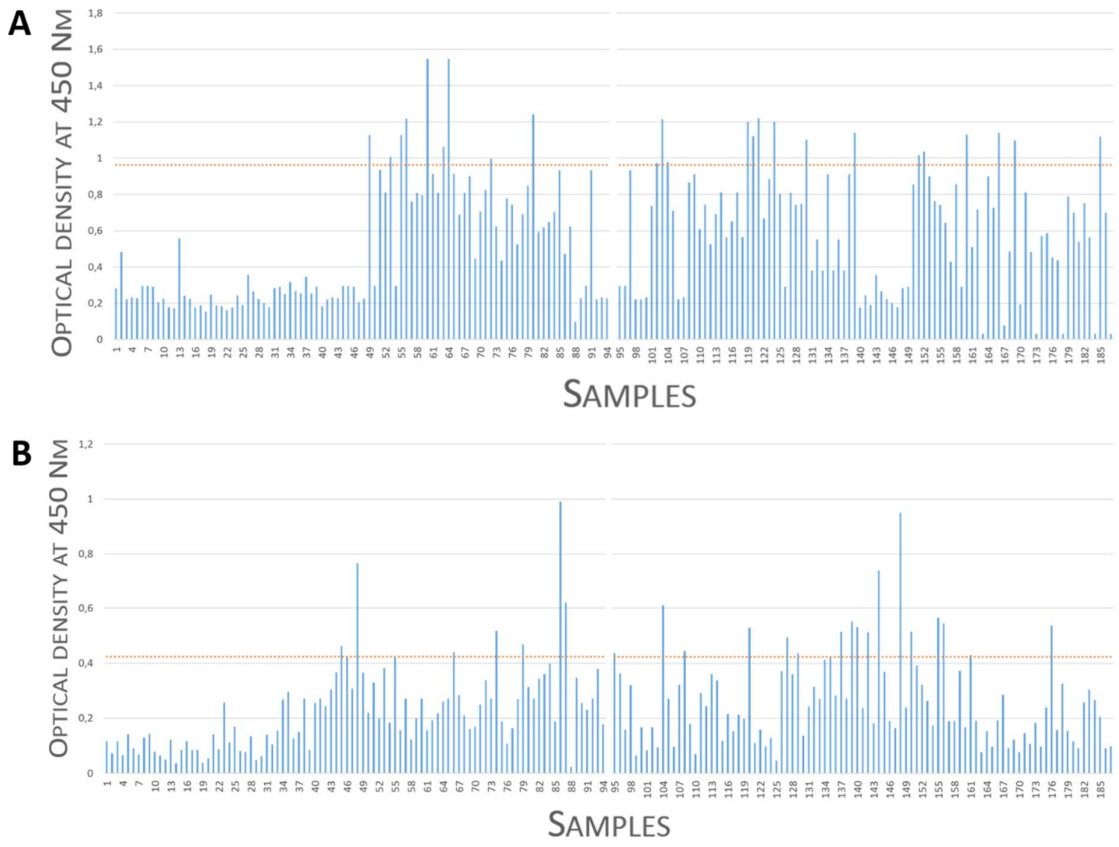


Fig. 44 Mean OD values of 187 serum samples tested negative by the YF microneutralization assay for the detection of A) IgG antibodies and B) IgM antibodies. The dotted orange line represents the individually calculated cut-off value

As is evident, the mean OD values for IgG detection were in general higher than for IgM detection.

The first step for calculating the cut-off value was to calculate the mean OD value of all negative serum samples that were tested. In the following step, the mean OD value of the blank wells was deducted from the mean OD value of the negative samples which is the mean OD_{Net}. Subsequently, the standard deviation (SD_N) of the mean OD_{Net} was calculated. The cut-off value was then taken as follows:

$$Cut - off\ value = mean\ OD_{Net} + SD_N$$

As all the negative samples were confirmed negative by micro-neutralization assay against YFV, the simple standard deviation was chosen over the two-fold and three-fold standard deviation.

For the evaluation of the samples, OD values above the calculated cut-off value were considered YFV IgG/IgM positive and below the cut-off value considered as negative.

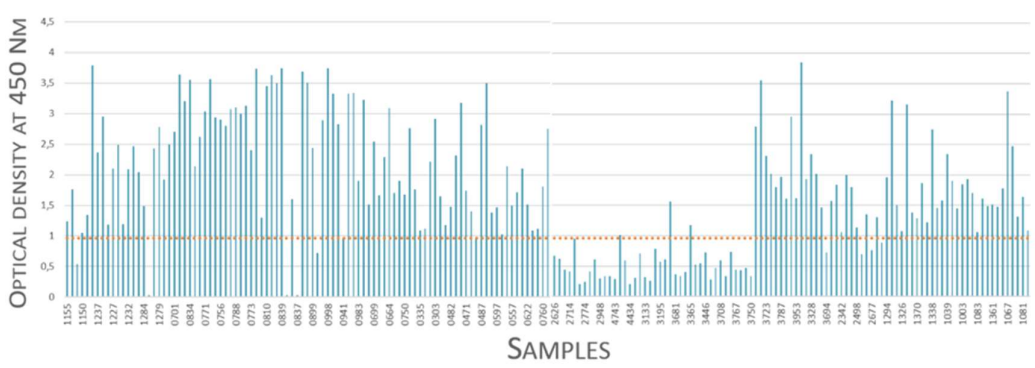
The cut-off value thus calculated for YFV IgG antibodies was 0.963 and for YFV IgM antibodies 0.424. These values were therefore used in order to evaluate the OD values for the 190 positive serum samples.

The following picture below depicts results of the indirect IgG ELISA.

Fig. 45 A illustrates results of the IgG ELISA in which all the serum samples have been tested for IgG levels against YFV. Each bar represents one serum sample. The orange dotted line is the calculated cut-off value of 0.963.

Fig. 45 B summarizes the results of Fig. 45 A in a table. As can be seen, of all the 190 tested serum samples (NT^+ - positive by microneutralization assay) for YFV IgG antibodies, 143 i.e. 75% were positive and 47 i.e. 25% were negative. Furthermore, in a second column, the analysis of the negative samples i.e. the samples tested negative by a microneutralization assay (NT^-) is presented. As can be seen out of 187 samples, 24 i.e. 13% were YFV IgG positive and 163 i.e. 87% YFV IgG negative.

A



B

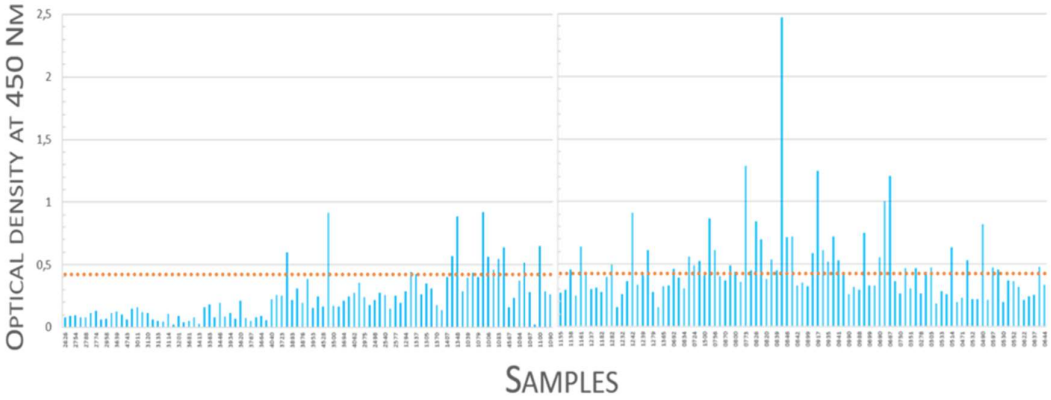
Number Of Analyzed Sera	190 (NT^+)	187 (NT^-)
IgG positive Sera	143 (75%)	24 (13%)
IgG negative Sera	47 (25%)	163 (87%)

Fig. 45 Results of the YFV IgG ELISA

A) Mean OD values of 190 serum samples of the PsA-TT 007 study tested positive by the microneutralization assay have been tested in this ELISA for IgG antibodies against YFV. The orange dotted line depicts the cut-off for positivity. Samples with OD values above the cut-off are considered positive and below the cut-off as negative. B) This table summarises the results obtained by this ELISA. A total of 190 serum samples positive by microneutralization assay (NT^+) and 187 serum samples negative by microneutralizatrion assay (NT^-) have been analyzed. Out of 190 tested samples 143 i.e. 75% are IgG positive and 47 i.e. 25% are IgG negative and out of 187 tested samples 24 i.e. 13% are IgG positive and 163 i.e. 87% are IgG negative.

The next figure (Fig. 46) presents results of the IgM YF-NS1 ELISA. The samples have been analyzed for their IgM levels against YFV and are represented as blue bars. The orange dotted line is the calculated cut-off value of 0.424 for this experiment.

A



B

Number Of Analyzed Sera	190 (NT^+)	187 (NT^-)
IgM positive Sera	57 (30%)	26 (14%)
IgM negative Sera	133 (70%)	161 (86%)

Fig. 46 Results of the YFV IgM ELISA
A) Mean OD values of 190 serum samples of the PsA-TT 007 study have been tested in this ELISA for IgM antibodies against YFV. The orange dotted line depicts the cut-off for positivity. Samples with OD values above the cut-off are considered positive and below the cut-off as negative.
B) This table summarises the results obtained by this ELISA. A total of 190 serum samples positive by microneutralization assay (NT^+) and 187 serum samples negative by microneutralizatrion assay (NT^-) have been analyzed. Out of 190 tested samples only 57 i.e. 30% are IgM positive and 133 i.e. 70% are IgM negative and out of 187 tested samples 26 i.e. 14% are IgM positive and 161 i.e. 86% are IgG negative.

As shown above in Fig. 46 A, the cut-off value of this ELISA is lower than the cut-off value of the IgG ELISA. In general, it is noticeable that the OD values of the samples are rather low when compared to the OD values of samples of the IgG ELISA.

Fig. 46 B summarizes the results of Fig. 46 A in a table. As can be seen, of all the 190 tested serum samples (NT^+ - positive by microneutralization assay) for YFV IgM antibodies, only 57 i.e. 30% were positive and 133 i.e. 70% were negative. Furthermore, in a second column, the analysis of the negative samples i.e. the samples tested negative by a microneutralization assay (NT^-) are presented. As can be seen out of 187 samples, 24 i.e. 14% were YFV IgM positive and 163 i.e. 86% YFV IgM negative.

In order to go into more depth and to see whether those IgG negative samples would be IgM positive or negative and vice versa, further data analysis was performed. The results are depicted in the picture below.

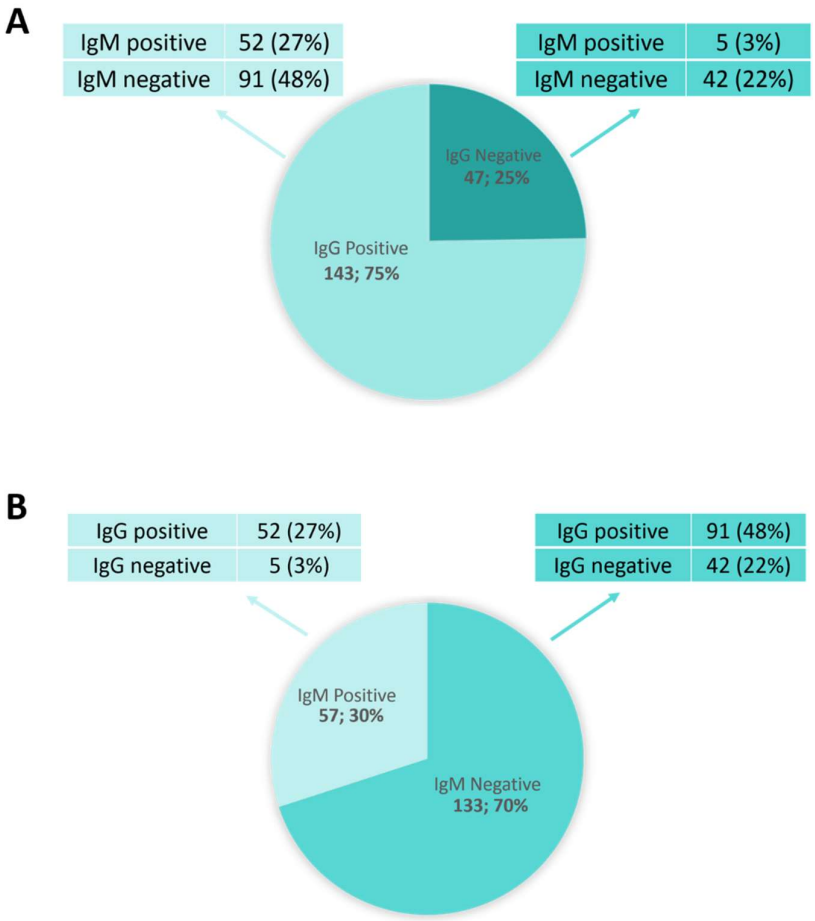


Fig. 47 Further analysis of IgG and IgM positive and negative samples.
A) The distribution between IgG positive and IgG negative samples is depicted in the middle by the pie chart. Out of the 143 IgG positive samples 52 (27%) are IgM positive as well and only 91 (48%) IgM negative. Out of the 47 IgG negative samples 5 (3%) are IgM positive and 42 (22%) samples are IgM negative as well.
B) The distribution between IgG positive and IgG negative samples is depicted in the middle by the pie chart. Out of the 143 IgG positive samples 52 (27%) are IgM positive as well and only 91 (48%) IgM negative. Out of the 47 IgG negative samples 5 (3%) are IgM positive and 42 (22%) samples are IgM negative as well.

The pie chart in the middle of Fig. 47 A depicts the distribution between the IgG positive and IgG negative samples. Out of the 143 (75%) YFV IgG positive samples, 52 (27%) were IgM positive as well and 91 (48%) IgM negative. When looking at the IgG negative samples, it is evident that 5 (3%) samples are IgM positive and 42 (22%) are IgM negative as well.

Consequently, the distribution of IgG positive and negative samples in IgM positive and negative samples can be derived. For a better understanding, this analysis is presented in Fig.

47 B. As can be seen out of the 57 (30%) YFV IgM positive samples, 52 (27%) were IgG positive as well and only 5 (3%) were IgG negative. Regarding IgM negative samples, 91 (48%) were IgG positive and 42 (22%) samples were IgG negative as well.

In order to see whether there is a correlation between *NT* values obtained by a micro-neutralization assay and OD values from the ELISA, results of the IgM and IgG ELISA were compared to the results of the micro-neutralization assay.

In the diagram below (Fig. 48) results of the comparison of the OD values of the IgG ELISA and their respective *NT* values are shown in a scatter plot. Herein, the *NT* value of each sample is plotted against its OD value.

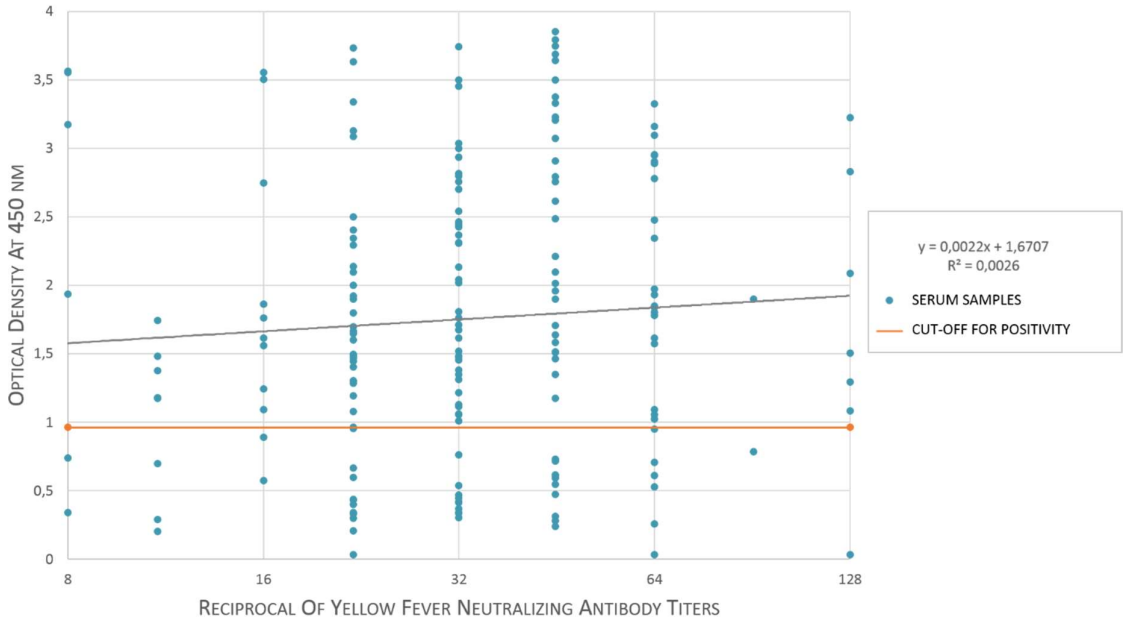


Fig. 48 Scatter Plot of IgG samples.
NT values are plotted against mean OD values of each sample. The blue dots represent the samples. The orange line marks the cut-off for positivity. The correlation coefficient R^2 was calculated by EXCEL and is shown in the box on the right-hand side.

As is noticeable in Fig. 48 OD values are scattered everywhere regardless of their corresponding titer values. Serum samples with a low *NT* value can have very high OD values of about 3.6. Serum samples with high *NT* titer values can have very low OD values of about 0.1. There is no correlation between OD value and *NT* value. This is confirmed by the very low correlation coefficient R^2 of 0.0026. Ideally, one would expect high titer values for corresponding high OD values and low titer values for corresponding low OD values.

The exact same data analysis was done for IgM samples and is presented in the next scatter plot (Fig. 49).

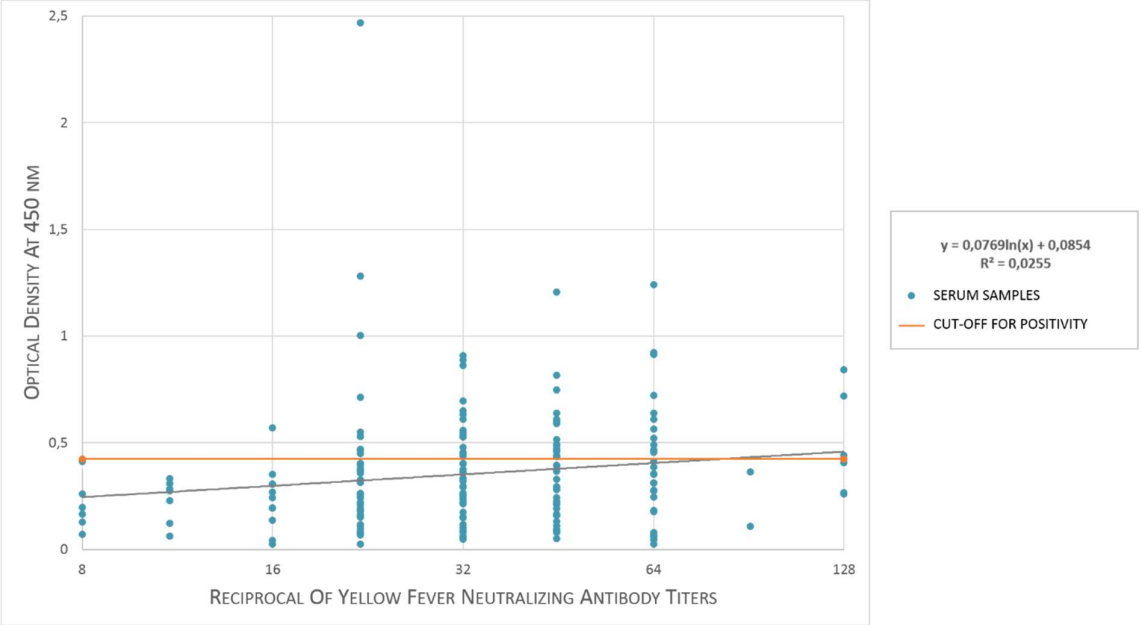


Fig. 49 Scatter Plot of IgM samples
NT values are plotted against OD values of each sample. The blue dots represent the samples. The orange line marks the cut-off for positivity. The coefficient of determination R^2 was calculated by EXCEL and is shown in the box on the right-hand side.

Interestingly, this scatter plot shows that most serum samples with low *NT* values (1:8 to 1:22) have also low mean OD values, which lie below the cut-off value. In contrast however, serum samples with a high *NT* value of 1:128 have very low OD values (< 1). Serum samples with *NT* values, which are in the middle, have OD values ranging from 0 to 2.5. Some samples with very high titer values have even OD values, which are below the cut-off value for positivity. The correlation coefficient R^2 has a value of 0.0255, which states that there is no correlation between *NT* value and corresponding OD value.

3.3 The xCELLigence™ System – Cellular Impedance Measurement

The aim of this project was to transfer standard virological methods like virus titration and neutralization assay on to the xCELLigence ™ system in order to see whether this would simplify the execution of the experiment and more importantly the subsequent evaluation. Hence two publications were chosen [98, 99] where these virological methods as well as

protocols had been developed. Witkowski et al. had established conventional techniques in pox virology whereas Fang et al. [98] had established these techniques for West Nile Virus and St. Louis encephalitis virus respectively. The differences in these publications, apart from the use of different viruses, were specific calculation methods for the quantification of neutralizing antibodies in samples. Therefore, these methods were adapted for YFV in order to see whether this would be a useful and worthwhile alternative to the conventional virological methods.

This chapter shows results, which were obtained by using methods from both the aforementioned publications. These methods had to be adjusted to the YFV at first. The first chapter shows results of a virus titration assay using the protocol and calculation methods established by Witkowski et al. [99]. The subsequent chapter depicts two different methods by Witkowski et al. [99] and Fang et al. [98] for quantifying neutralizing antibodies against YFV in serum samples.

The initial steps of establishing a protocol on virus titration and neutralization assay was part of the author's diploma thesis and hence not shown here. There, the determination of the concentration of each component, like cells, virus and serum was of great importance and could be established. For the determination of an optimal cell density, different concentrations of two different cell lines, PS cells and Vero cells, were seeded into an E-plate 96, which is coated with microelectrodes. Thereafter the impedance was determined as cell index (CI_x) value and cell impedance was measured every 10 minutes producing different cell proliferation graphs. The cell growth was monitored using the Real-Time Cell Analyzer (RTCA) SP Instrument. The results showed that PS cells compared to Vero cells in the concentration of 10.000/well gave the best performance in regard to the course of the graph – i.e. a steady increase at the beginning and a steady decrease at the end. Hence, for all following experiments 10.000 PS cells/well, which is 100 µL of cell solution per well were employed.

All the experiments were run in duplicates and the values used for depicting the proliferation curves are the mean of two readings. There is a deviation from this double determination only in those cases, where specifically mentioned.

3.3.1 YFV-17D titration

For the quantification of virus particles in any sample the method used was established by Witkowski et al. [99]. For this, ten-fold serially diluted virus preparations of a YFV-17D viral stock of known titer was employed in parallel to four unknown YF-17D virus stocks in order to determine the titer of these.

As already mentioned the YFV-17D virus stock with known titer was used as a standard. Using this stock, ten-fold serial dilutions were prepared in quadruplicates in a total volume of 50 µL. One hundred µL of 10,000 PS cells/well were added to each viral dilution and the experiment was run for 140 hours. Fig. 50 depicts the curves obtained for each virus dilution. The green curve represents the cell control curve, which shows a steady increase in the CI_x value to approximately 12.9 till the experiment ends. The remaining curves represent the respective virus dilutions.

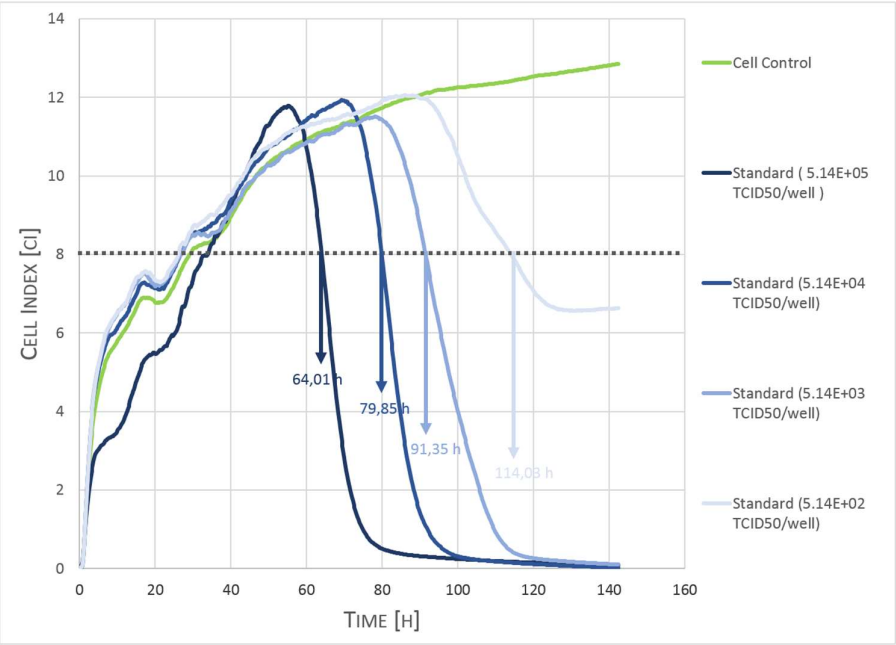


Fig. 50 Titration of YFV-17D virus stock as standard to determine the titer of four different virus stocks
10000 PS cells per well were infected with 4 different concentrations of a YFV-17D virus stock in quadruplicates. Cells were monitored for 140 h. A threshold at a definite CI value, which covers the range of every virus concentration, was set at CI = 8 (marked by the dotted black line). The time point of intersection of each curve with CI = 8 was utilized to generate the standard curve, which is depicted in Fig. 51

As shown in Fig. 50, each of the blue curves shows a similar progress in contrast to the cell control. The sharp increase of the CI_x value takes place in the initial phase during the first 50 hours in the phase of cell growth. Thereafter each curve drops to a CI_x of zero at definite time points depending on the virus dilution. The dark blue curve depicts the lowest virus dilution, whereas the light blue curve shows the highest virus dilution. As it can be seen, the dark blue curve starts to descend at approximately 50 h and the light blue curve at about 90 h.

To generate the standard curve a threshold at a definite CI_x value has to be set at first, where the distance of each dilution curve is approximately the same. This means that every experiment will have its own threshold. Here we set the CI_x threshold at 8, depicted as a black dotted line in Fig. 50. At this threshold, the time points of each virus dilution curve were taken and plotted against the respective $TCID_{50}$ concentrations. The time points taken for the generation of the standard curve are marked with arrows and are 64.01 h, 79.35 h, 91.35 h and 114.03 h.

Fig. 51 shows the standard curve of the virus stock of known titer, which has been used as standard. The standard curve was generated by a linear regression through the four determined time points. Thereby R^2 amounted to be 0.982 and the graph resulted in the linear equation of:

$$t_{(CI_x=8)} = -7.018 \ln(c) + 139.19$$

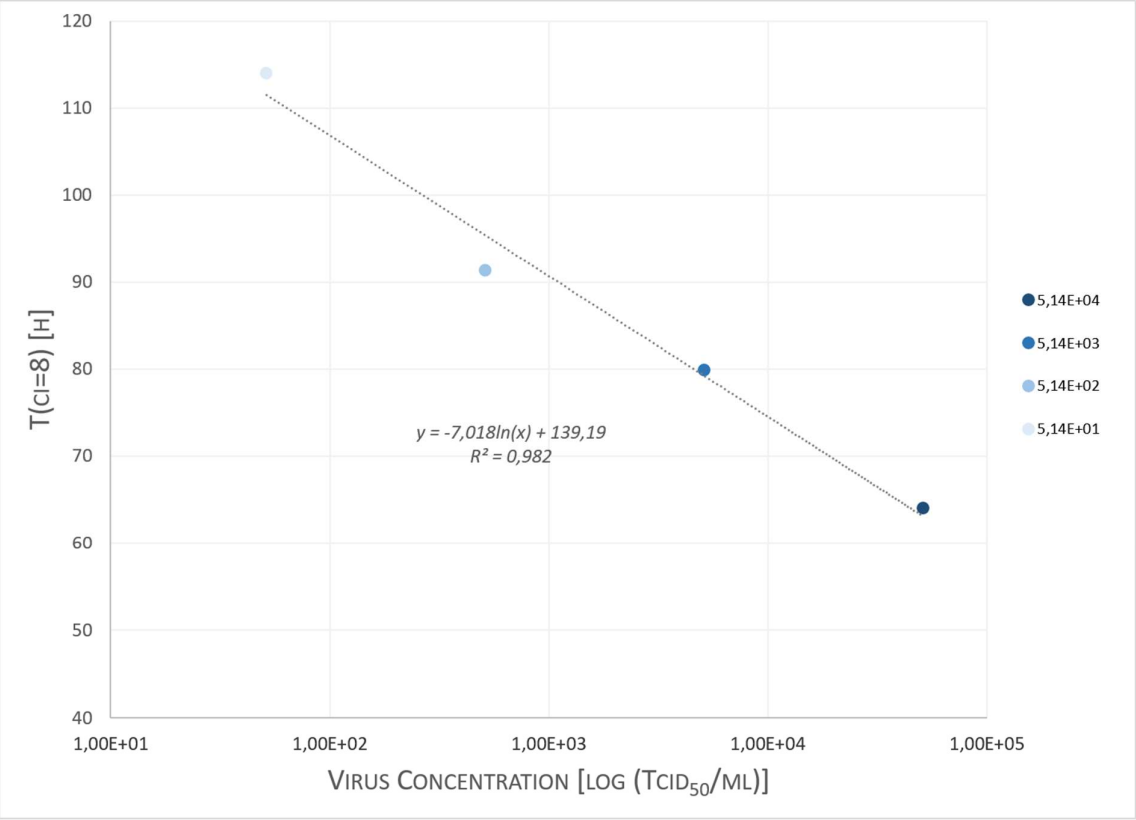


Fig. 51 Standard Curve of the virus stock of known titer
The time points of intersection of each curve are plotted in a logarithmic manner against each virus concentration. The equation of slope is utilized to calculate the titer of the unknown virus stocks. The correlation coefficient of the curve equates to R^2= 0.982.

With the help of this equation, the titer of the unknown virus stocks could be calculated. As an example, the titer of virus stock A (see Fig. 46) is calculated.

The equation is solved for *c*, which is the concentration of the unknown virus stock A, resulting in the following equation:

$$c = e^{\left(\frac{t(CI_x=8)-139.19}{-7.018}\right)}$$

where t(CI_x =8) is the time point at CI_x =8 of the unknown YFV-17D virus stock A. Fig. 52 shows the curves for the unknown virus stocks A, B, C and D. Of each virus stock, a 1:10 virus dilution was prepared in quadruplicates. The time point was determined by setting the threshold at CI_x =8, marked by the black dotted line in Fig. 52. The intersection of the black line and the curve for the virus dilution 10^{-1} marked with a green arrow resulted in the time point t(CI_x =8) = 64.01 h.

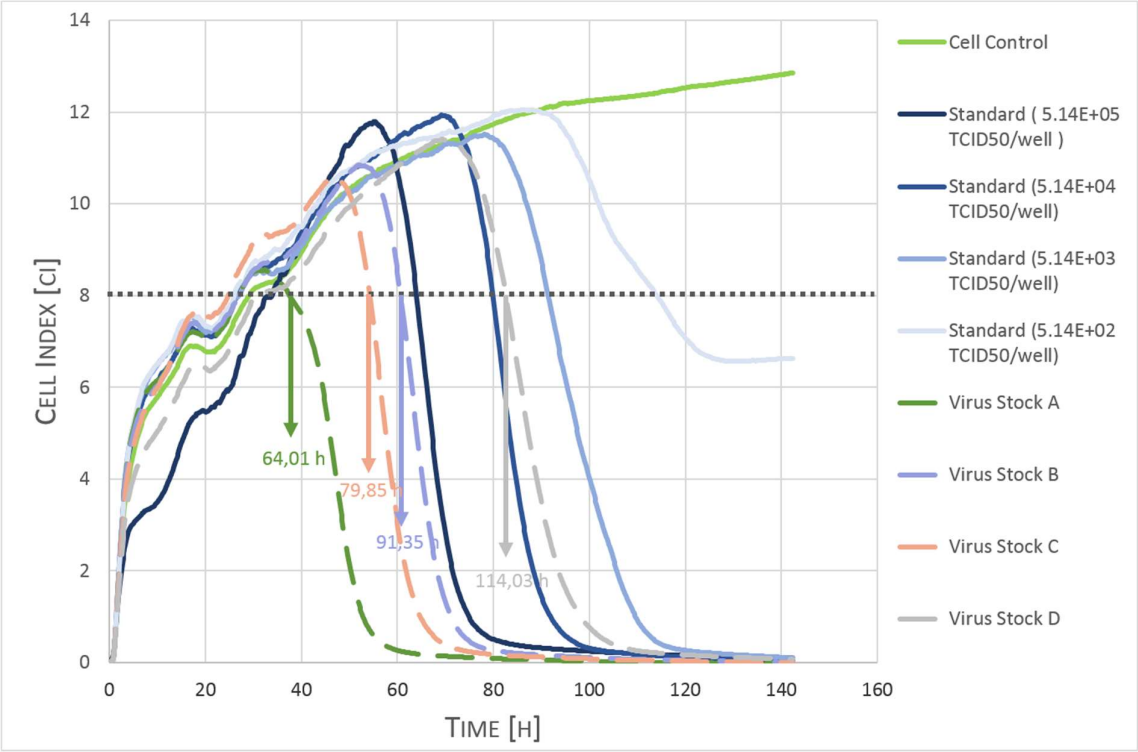


Fig. 52 Determination of the titer of virus stock A

10.000 PS cells per well were infected with a 10^{-1} virus dilution in quadruplicates. As control only PS cells were seeded. The cells were monitored for 140 h. To calculate the virus titer the time point of intersection of *CI* =8 with the curve with the infected cells (marked with arrows) is used. The respective time point is then employed in the equation of the slope of Fig. 51 and the virus titers of the unknown virus stocks are calculated.

This value is then taken for solving equation above in order to calculate the titer, which results in 1.96E+06 $TCID_{50}$/mL. Since the virus dilution is not considered in the calculated titer, this result has to be divided by the dilution factor 0.1 (=10^{-1}) to get the final titer of the YFV-17D virus stock A, which is then 1.96E+07 $TCID_{50}$/mL. The remaining YFV-17D virus stocks B, C

and D were calculated the same way. In order to verify the results, the unknown viral stocks have been titrated by the conventional virus titration assay as well. All the results are depicted in the following table.

Table 13 Results of the virus titration assay on the xCelligence system (RTCA) and the conventional microtiter assay

SAMPLES	Titer by RTCA $TCID_{50}$/ml	Titer by Microtiter Test $TCID_{50}$/ml
Virus Stock A	1,96E+07	5,44E+07
Virus Stock B	7,23E+05	1,31E+06
Virus Stock C	1,83E+06	5,32E+06
Virus Stock D	3,22E+04	7,51E+04

As can be seen the virus titer obtained by the conventional microtiter assay is slightly higher than the virus titer obtained by the xCELLigence™ system. However, as the difference is less than one log this difference can be neglected.

3.3.2 Microneutralization assay

The execution of the microneutralization assay on the xCelligence™ system was similar to the usual neutralization assay. Two-fold serial serum dilutions were prepared in duplicates in a total volume of 25 µL and mixed with 25 µL virus dilution. This blend was incubated for 1 h at 37°C and thereafter 100 µL of 10.000 PS cells/well were added to each well (chapter 2.7.2). The cells were monitored for 140 h to 160 h. As already mentioned above in this chapter, the two different mathematical methods for quantifying neutralizing antibodies against YFV in serum samples were tested. The first method presented here is the method by Witkowski et al. [99].

A total of nine sera were tested for neutralizing effects in regard to YFV-17D. All of these serum samples were from YFV-17D vaccinated people and have been pre-tested in the classical microneutralization assay. Each experiment included the serum sample in different dilutions, a cell control (only cells), serum control (cells + serum), virus control (cells + virus) and negative control (serum of non-vaccinated person). Note that only the serum control and the virus control have been included in the graphics in order to simplify it. In general, only those experiments where all the controls gave the expected results have been considered as successful and hence have been evaluated.

In Fig. 53 graphs of sample 4 are shown. The green curve is the serum control (uninfected 1:8) and the red curve the virus control. ‘Uninfected 1:8’ means that serum has been 1:8 diluted and

then added to the cells. In the virus control, virus particles have been added to the cells to reach 100 $TCID_{50}$/well. The remaining curves depict the respective serum dilutions. As can be seen, there is a clear correlation between the serum dilution and the cell index over the time – the lower the serum dilution the higher the cell index of each curve and vice versa. Furthermore, the lower the serum dilution the longer the time till the cell index starts to decrease, which is a sign for cell death. Hence, the 1:8 serum dilution shows minimum decrease, whereas serum dilutions 1:16 and 1:32 show a decrease in the cell index after approximately 120 hours. The cell index of serum dilution 1:64 decreases after approximately 80 hours and that of serum dilution 1:128 after 70 hours. Furthermore, the CI_{NT50}, which is the CI_x value reflecting 50% neutralization of the virus, is depicted in the figure for each time point (black dotted line).

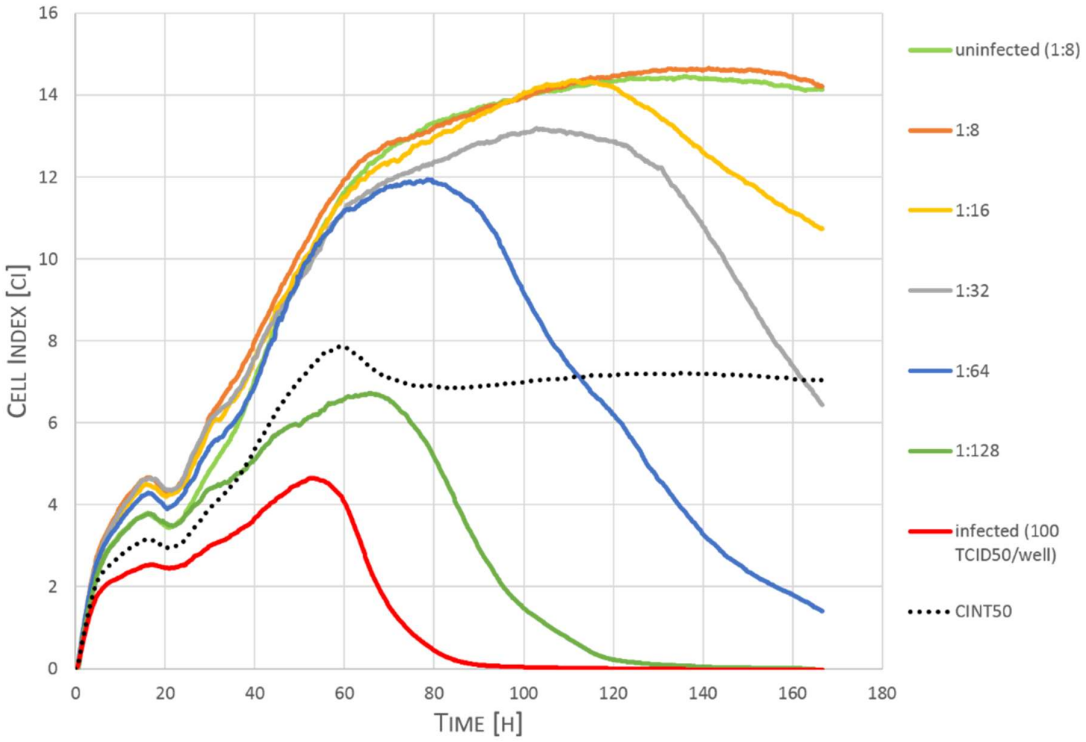


Fig. 53 Neutralization assay for sample 4
Two-fold serial dilutions of serum sample 4 were incubated with virus of a concentration of 100 $TCID_{50}$/well for one hour at 37°C. This mixture was then inoculated with 10.000 PS cells per well. The *CI* development was monitored for 170 h. The light green curve represents the serum control, where 1:8 diluted serum has been incubated with cells and the red curve depicts the virus control, where 100 $TCID_{50}$/well has been used. The remaining curves show the different serum dilutions. It is noticeable that the maximum CI values of each curve correlated with the respective serum dilution. Only serum dilution 1:8 showed neutralizing activities almost till the end of the experiment. The remaining dilutions started to descent according to their dilutions indicating the time point where the serum dilutions had no neutralizing properties anymore. Furthermore, the CI_{NT50} line is depicted (black dotted line). The CI_{NT50} value reflects 50% neutralization and was calculated for each time point.

To determine the CI_{NT50} for each time point, following formula was employed:

$$CI_{NT50} = \frac{1}{2}(CI_U - CI_V) + CI_V$$

In this CI_U stands for the cell index of the curve for uninfected serum control and CI_V denotes the cell index of the virus control. Both the values were taken at each time to calculate the course of the curve, which would reflect 50% neutralization at each time point.

In order to quantify neutralizing antibodies against YFV in serum samples, determined CI_x values and corresponding serum dilutions were plotted in a logarithmic manner to generate a standard curve. With the help of the equation for the linear slope of the graph, the serum dilution, which corresponds exactly to CI_{NT50}, could be calculated. The time point for the determination of the CI_x values should lie in the exponential death phase of the virus infected cells. Furthermore, a time point should be chosen, where when plotted in a graph, the correlation coefficient comes as close to one as possible as this would indicate a positive linear relationship between the data. Thus, each experiment has a different time point of determination. For our experiment, we set the time point of determination (t) to 160 hours. For the generation of the standard curve we determined CI_x values of each serum dilution at 160 h. The determined CI_x values for sample 4 are presented in the next figure Fig. 54.

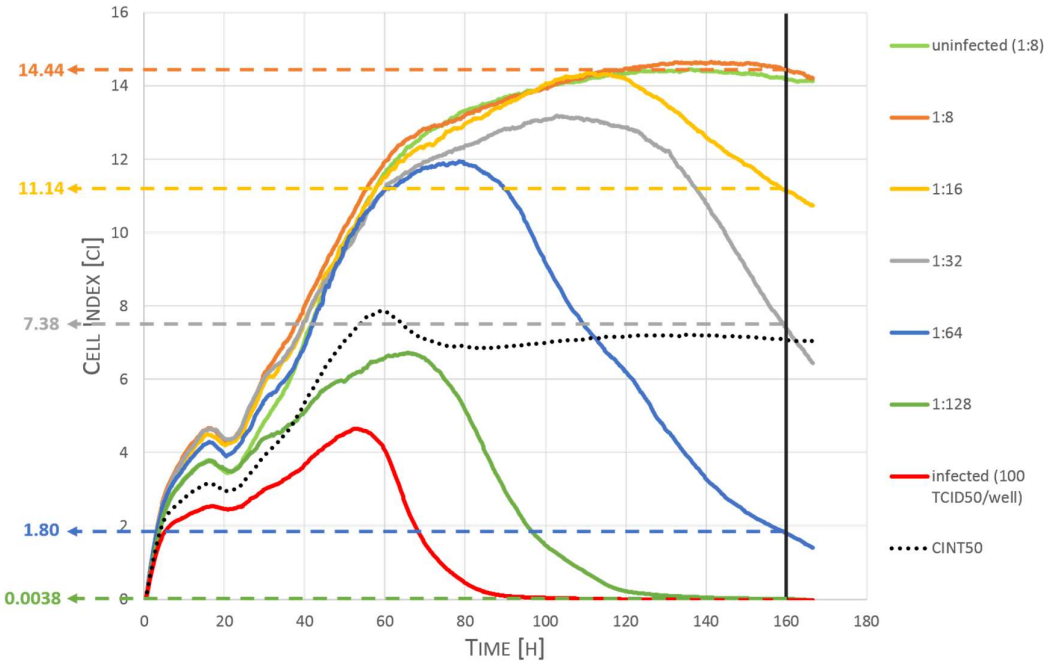


Fig. 54 Determination of CI values at 160 h for quantification of neutralizing antibodies in sample 4
For a quantitative determination of the serum titer a time point (t) was set 160 h and the corresponding CI values of each serum dilution taken to generate a standard curve (depicted in Fig. 55)

As already mentioned the determined CI_x values at 160 h are plotted in a logarithmic manner to generate a standard curve. This curve is shown in the next figure (Fig. 55) with the slope equation and the correlation coefficient R^2.

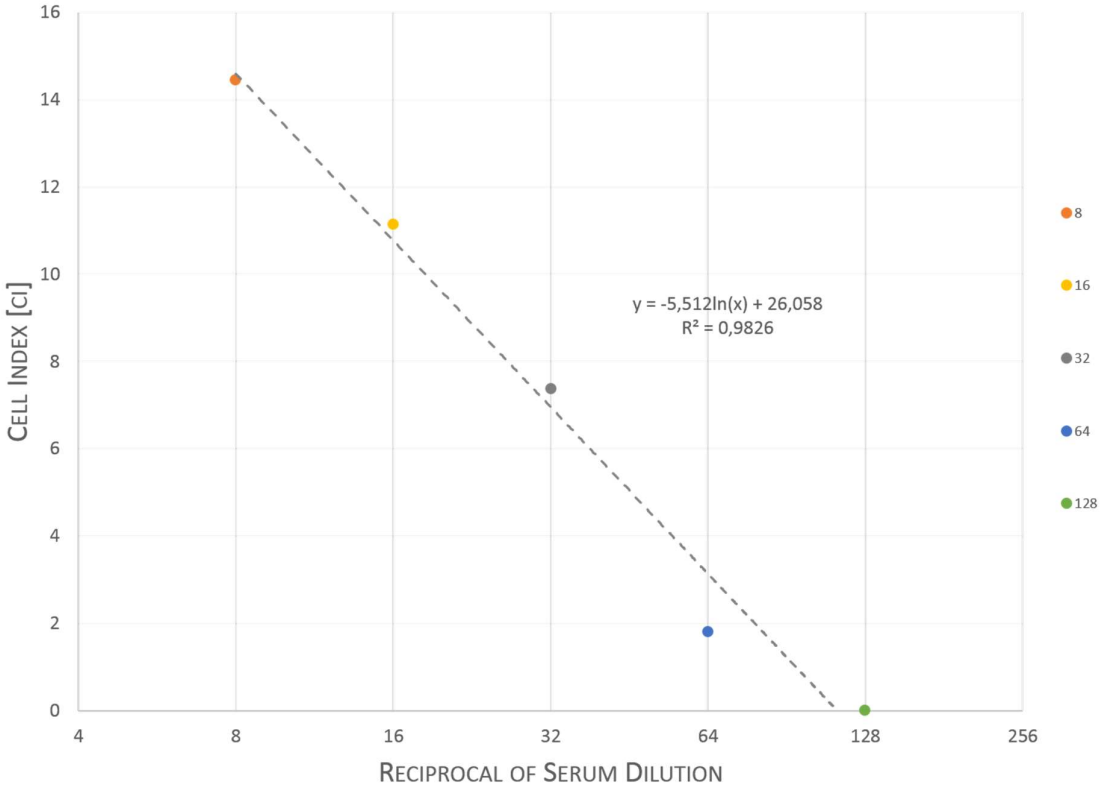


Fig. 55 Standard curve of sample 4
CI values, which were determined at 160 h, were utilized to generate this standard curve for sample 4. The slope equation was solved for x to calculate the quantitative serum titer of the sample at 50% virus neutralization. The colored dots represent the respective reciprocal [1/x] serum dilutions.

To calculate the serum dilution of sample 4 corresponding to CI_{NT50}, the slope equation was transformed to

$$qRTNT_{50} = e^{((CI_{NT50}-26.058)/-5.512}$$

Where $qRTNT_{50}$ the quantitative real-time neutralization titer, denotes x and CI_{NT50} stands for y. The CI_{NT50} value at this specific time point amounted to be 7.1. With this value, this equation has been solved and resulted in a quantitative neutralization titer of 1:31. The remaining eight serum samples have been calculated the same way and the results are presented in the following table (Table 14) together with the *NT* results obtained by the conventional microneutralization assay.

Table 14 Titer Values of nine serum samples
Shown are titer values obtained by the method of Witkowsky et al. (*qRTNT$_{50}$*) and the conventional microneutralization assay (*NT*). *qRTNT$_{50}$* values, which are in red, lie above or below the range of acceptability of two dilutions of the corresponding *NT* value

SAMPLE	NT_{50}	$qRTNT_{50}$
1	1:16	**1:75**
2	1:1024	1:636
3	1:25600	1:27031
4	1:32	1:31
5	1:256	1:80
6	1:256	**1:39**
7	1:256	1:134
8	1:512	1:515
9	1:128	1:96

As can be seen, sample 1 shows a difference in the titer values between *NT* and *qRTNT$_{50}$* of more than 2 dilutions. The same can be seen with sample 6 (marked in red). For sample 2, 3, 7 and 9 the difference between *NT* and *qRTNT$_{50}$* lies within one dilution or less. Sample 4 and sample 8 have hardly any difference in their titer values.

In order to investigate assay to assay variation sample 2 was tested by this method four times and samples 3, 4 and 7 two times. The results are shown in the following table (Table 15) together with the *NT* results.

Table 15 Assay to assay variation
Sample 2 was tested over a period of four days and sample 3, 4 and 7 over a period of 2 days in order to analyze the assay to assay variation.

SAMPLE	NT_{50}	$qRTNT_{50}$				
2	1:1024	1:636	1:2940	1:428	1:484	1:277
3	1:25600	1:27031			**1:214015**	
4	1:32	1:31			1:64	
7	1:256	1:134			1:668	

As is shown, the range of variation in *qRTNT$_{50}$* between each titer value for sample 2 and 7 lies within two dilutions above and below the corresponding *NT* value. This cannot be seen for sample 3, where the second titer value in *qRTNT$_{50}$* has a difference of more than 2 dilutions above the corresponding *NT* value (marked in red). The range of variation in *qRTNT$_{50}$* between the two titer values for sample 4 lies within one dilution above the corresponding *NT* value.

The next method presented here is by Fang et al [98]. The interesting part of this method is the single well detection of neutralizing antibodies against a virus in serum samples with the help of a standard, where the antibody titer is known. Hence, all the serum samples to be tested can be run in parallel to the standard.

For the determination of the neutralizing titer in the serum samples specific time points at the 50% decrease of the CI_x of the lowest serum dilution, the CIT_{50} value, are plotted against the corresponding antibody titer of the standard in a logarithmic manner to generate a linear regression curve. With the help of the equation for the linear slope of the graph, serum titers corresponding exactly to the CIT_{50} can be calculated.

The execution of the assay remained the same. Hence, the first step was to establish different standards with known antibody titers. For this, different serum samples, which have been pre-tested by the classical microneutralization assay have been tested in different dilutions. The aim here was to obtain curves, which were proportionally equidistant to each other. We were able to establish 5 different serum samples as standards. Four of the five serum samples are presented figure Fig. 56. All the controls as previously used were included in each experiment. Only the curves for serum control (uninfected 1:8) and virus control beside the serum dilutions are shown here. As the CIT_{50} value is important for the quantification of neutralizing antibody titers of the unknown samples, it has been calculated individually for each experiment and included in the graphics.

Fig. 56 shows four different serum standards, which have been serially two-fold diluted ranging from 1:8 to 1:4096. As can be seen all serum standards show a linear relationship between serum dilutions and the time of decrease after the initial phase of cell growth. However, the curve patterns of each sample look different. Fig. 56 A shows a decrease in the CI_x value for every dilution whereas in Fig. 56 B the lowest serum dilution of 1:8 shows no decrease at all. This can be seen for the standards shown in Fig. 56 C and Fig. 56 D as well. Once the standards have been established, the unknown serum titers of the serum samples could be calculated. A total of 18 serum samples have been analyzed in regard to their neutralizing antibody titer against YFV. For a better understanding of the method, the calculations of neutralizing antibody titers of four different serum samples are shown in the following. The standard used for the calculation of these samples is shown in the next figure (Fig. 57). Nine serum dilutions ranging from 1:120 to 1:30720 have been employed. As is clearly visible, the serum control shows no decrease in the CI_x value whereas the virus control shows a sharp decrease in the CI_x value after approximately 70 hours.

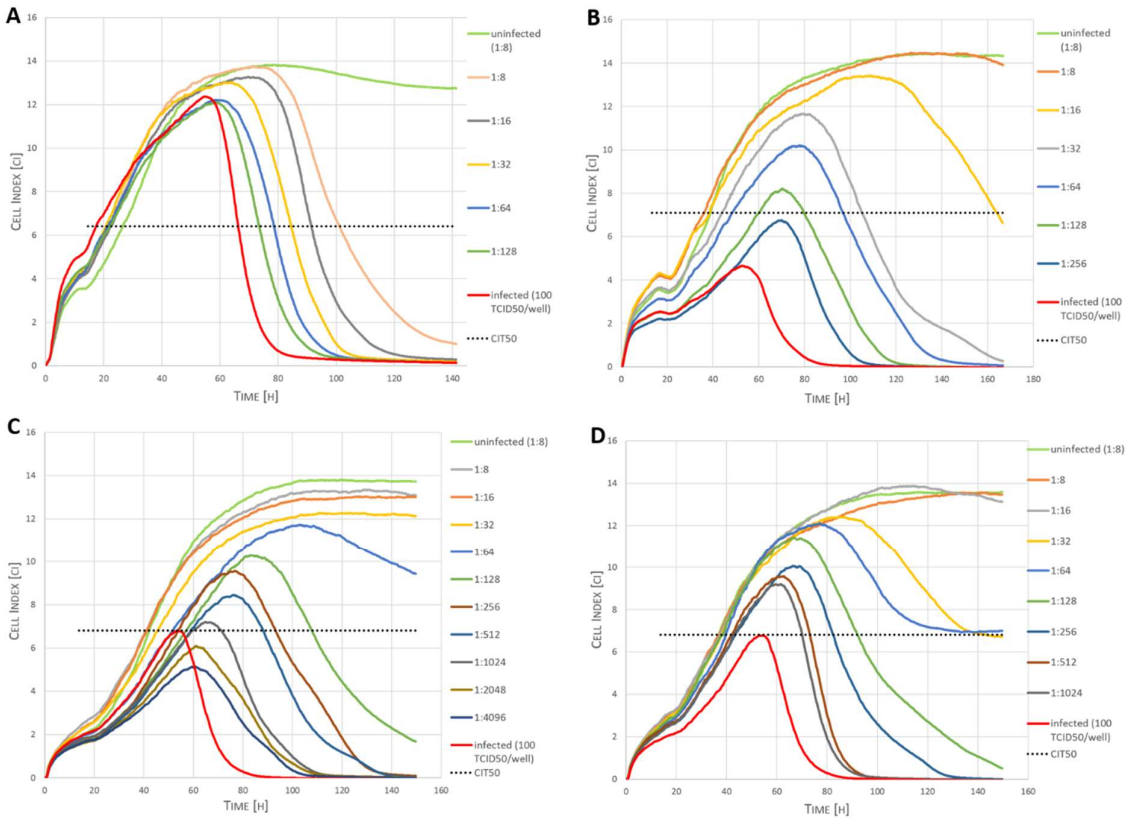


Fig. 56 A-D Neutralizing activities of different serum dilutions of the four serum standards A to D
Two-fold serial dilutions of each serum sample were incubated with virus of a concentration of 100 $TCID_{50}$/well for one hour at 37°C. This mixture was then inoculated with 10.000 PS cells per well. The CI_x development was monitored for 140 h to 170 h. The light green curve in Fig. 56 A to D represents the serum control, where 1:8 diluted serum has been incubated with cells. The red curve in those images represents the virus control, where 100 $TCID_{50}$/well have been used. The remaining curves show the different serum dilutions. Furthermore, the CIT_{50} line is also shown (black dotted line), which reflects 50% of the *CI* value of the lowest serum dilution (1:8). Hence, each standard has an individual CI_{NT50} value. All serum standards show a linear relationship between serum dilutions and the time of decrease.

For the calculation of the neutralizing antibody titer of the unknown serum samples only the first five serum dilutions (1:120 to 1:1920) have been used as the remaining serum dilutions were not equidistant to each other. The time points (marked with an arrow) of these serum dilutions at a specifically for this standard calculated CIT_{50} value of 5.8 (black dotted line) have been plotted logarithmically against the known antibody titers to generate a regression curve, which is shown in Fig. 58. As the mathematical model is based upon known antibody titers of the standard, the respective serum dilutions, which have been used for the regression curve have to be considered in the calculation. This standard had a *NT* value of 1:1024. Hence, this titer had to be divided by the respective serum dilution to obtain the actual antibody titer after the dilution. For the serum dilution of 1:120 this would mean as follows:

$$Ab_{titer} = 1024/120$$

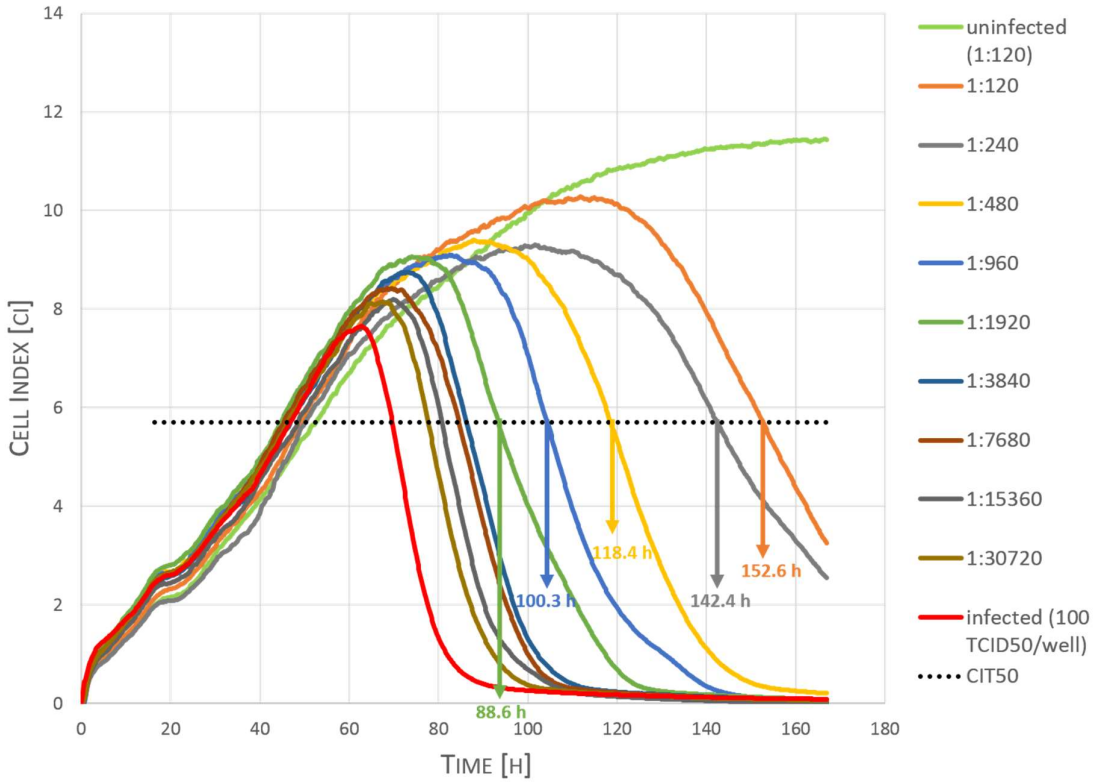


Fig. 57 Neutralizing activity of a Serum used as a standard and determination of time points of the different dilutions at the CIT_{50} value
In order to calculate unknown serum titers of serum samples, definite time points (marked with an arrow) of the respective dilutions of the standard at a specific CIT50 value were plotted logarithmically against each serum dilution to generate a linear regression curve (depicted in Fig. 58)

This results in an antibody titer of 8.5 for the standard in the serum dilution 1:120. The antibody titers of the remaining serum dilutions have been calculated the same way.

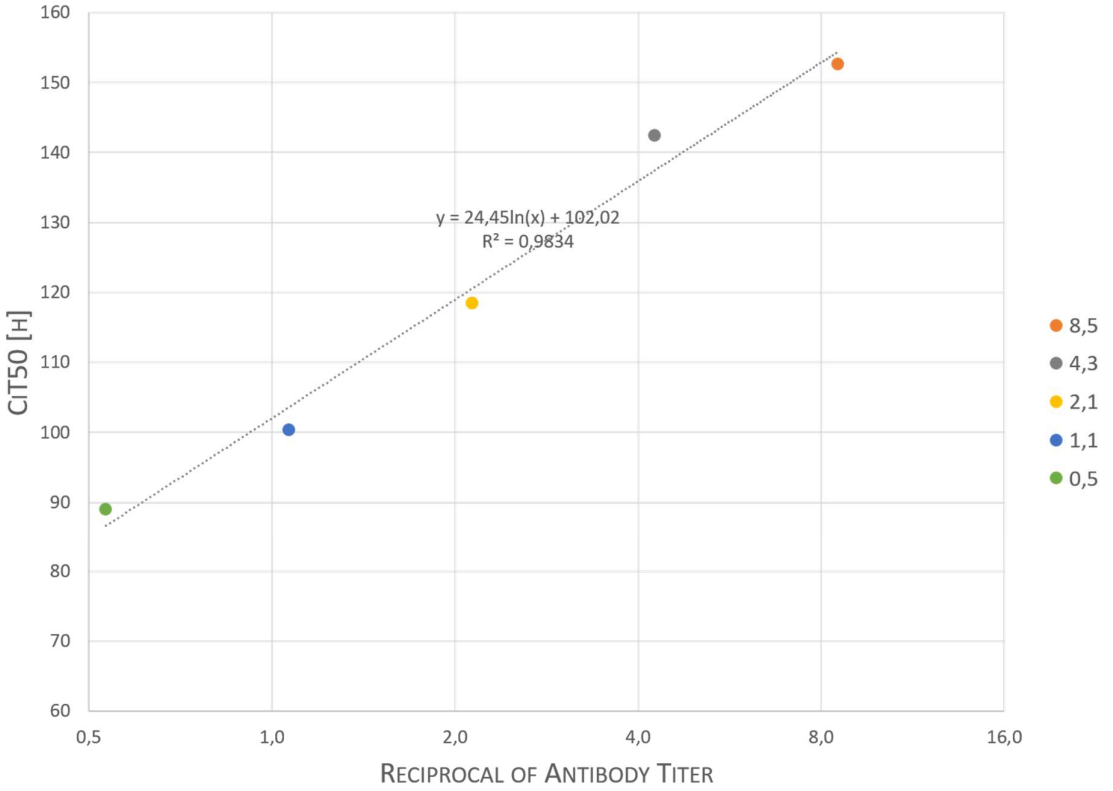


Fig. 58 Linear regression curve of the standard shown in Fig. 57
Definite time points, which were determined at a specific CIT_{50} value, of the corresponding serum dilutions were utilized to generate this linear regression curve. The slope equation was solved for x to calculate the quantitative serum titer of the sample. The colored dots represent the respective antibody titer of each serum dilution.

In order to calculate the antibody titer of the samples the slope equation was transformed to

$$RTCAT_{50} = e^{((CIT_{50}[h]-10\ \ .02)/24.45)}$$

where $RTCAT_{50}$, the real-time cell analysis titer reflecting 50% neutralization of the virus, denotes x and $CIT_{50}[h]$ stands for y. The $CIT_{50}[h]$ value is the time point of intersection between the unknown serum sample and the CIT_{50} value. The next figure Fig. 59 shows the standard together with four different serum samples. The different time points of intersection with the CIT_{50} value of the four different samples have been included in this figure (Fig. 59). For the purposes of illustrating this, the calculation of the neutralizing antibody titer of sample 11 has been shown. As shown below, the time point of intersection with the CIT_{50} value for sample 11 is 90.8 h. Hence, with this value the equation has been solved and resulted in a real-time cell analysis titer of 1:0.6. However, as this sample has been diluted to a ratio of 1:120, the result has to be multiplied by the dilution factor of 120 and therefore resulted in a real-time cell analysis titer of 1:76.

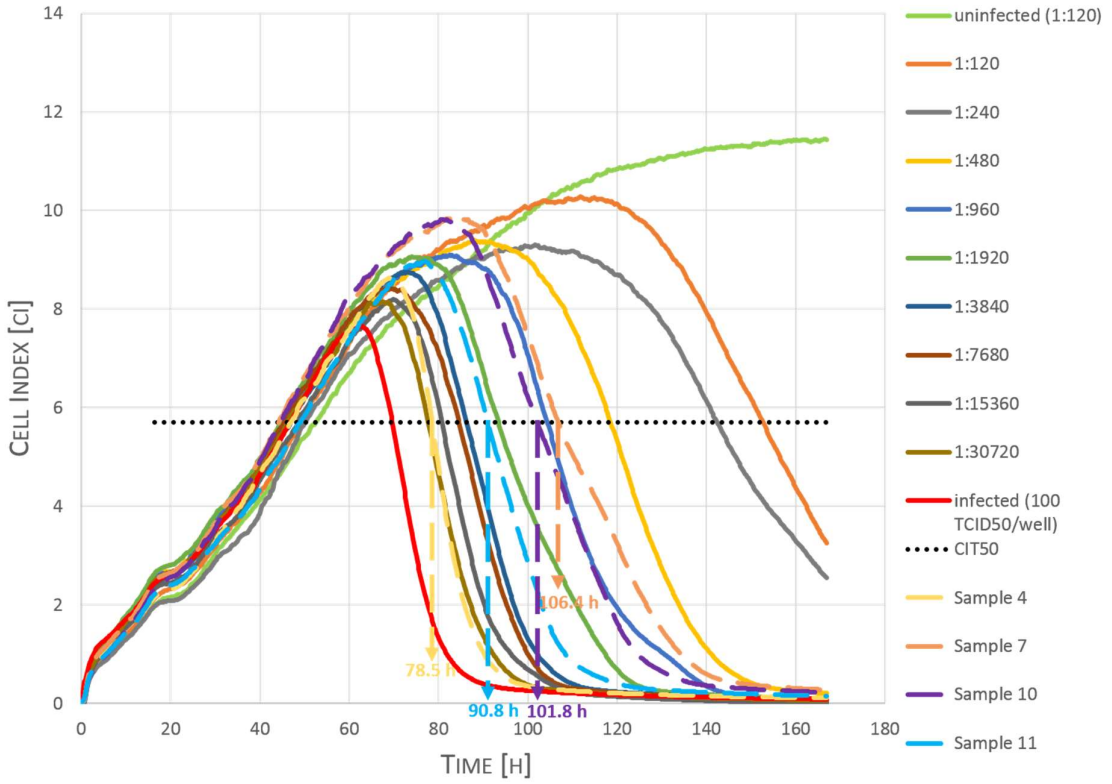


Fig. 59 Determination of the antibody titer of sample 11
Neutralizing activities of the standard together with to-be-tested samples. To calculate the antibody titer of sample 11 the time point of intersection of the CIT_{50} value with the curve of sample 11 is used. The respective time point is then employed in the equation of the slope of Fig. 58 and the antibody titers of the samples are calculated.

The antibody titers of a total of 20 serum samples have been determined in exactly this way. All these samples have been pre-tested by the microneutralization assay and hence have known *NT* values. The results are presented in the following table (Table 16). Some of the samples have been tested more than just one time and hence have more results. In order to evaluate the results and the method correctly, the same acceptability criteria was used as previously. Therefore, all results which had a difference of more than two dilutions above or below the *NT* value were considered as not valid. These results are marked in red. Only 3 out of 20 samples (sample 1, 2 and 3) have one titer value that is not valid. However, sample 1 and sample 2 have been tested more than just once and all the other results for these fall in the range of acceptance. Sample 3 has been tested only once and the result doesn't lie within the range of two dilutions.

Table 16 Antibody Titer values, which reflect 50% virus neutralization of 20 serum samples
Shown are titer values obtained by the method of Fang et al ($RTCAT_{50}$) and the microneutralization assay (*NT*). $RTCAT_{50}$ values, which are in red lie above or below the range of acceptability of two dilutions of the corresponding *NT* value. Those samples, which have values in green, could not be calculated by this method of Fang et al. It could be seen that these samples have neutralizing activity at a serum dilution of >1:8

SAMPLE	NT_{50}	$RTCAT_{50}$					
1	1:16	1:1	1:8	1:51			
2	1:1024	1:54	1:458	1:629	> 1:8	1:3781	
3	1:25600	1:128601					
4	1:32	1:23	1:50	1:46	1:50	1:57	
5	1:256	1:194	1:257	1:371	1:656	1:534	
6	1:256	1:304	1:437	1:681			
7	1:256	1:134	1:152	1:758	> 1:8	1:144	1:263
8	1:512	1:343	1:812	1:1429			
9	1:128	1:106					
10	1:128	1:181	1:417	1:119			
11	1:32	> 1:8	1:76				
12	1:64	1:126	1:186	1:204	1:236	1:162	
13	1:128	1:168	1:251	> 1:8			
14	1:45	1:97	1:145				
15	1:256	> 1:8					
16	1:8	> 1:8					
17	neg	1:4					
18	1:1024	> 1:8					
19	1:256	> 1:8					
20	neg	1:7	< 1:1				

What is striking is that only 3 samples, namely sample 4, 9 and 13 have titer values, which are in the range of one dilution above or below the respective *NT*. The titer values of the remaining 14 samples lie in the range of maximum two dilutions above or below the respective *NT* values. As is evident some of the samples don't have actual titer values but just an above one to eight (> 1:8). The reason for this is depicted in the following figure (Fig. 60).

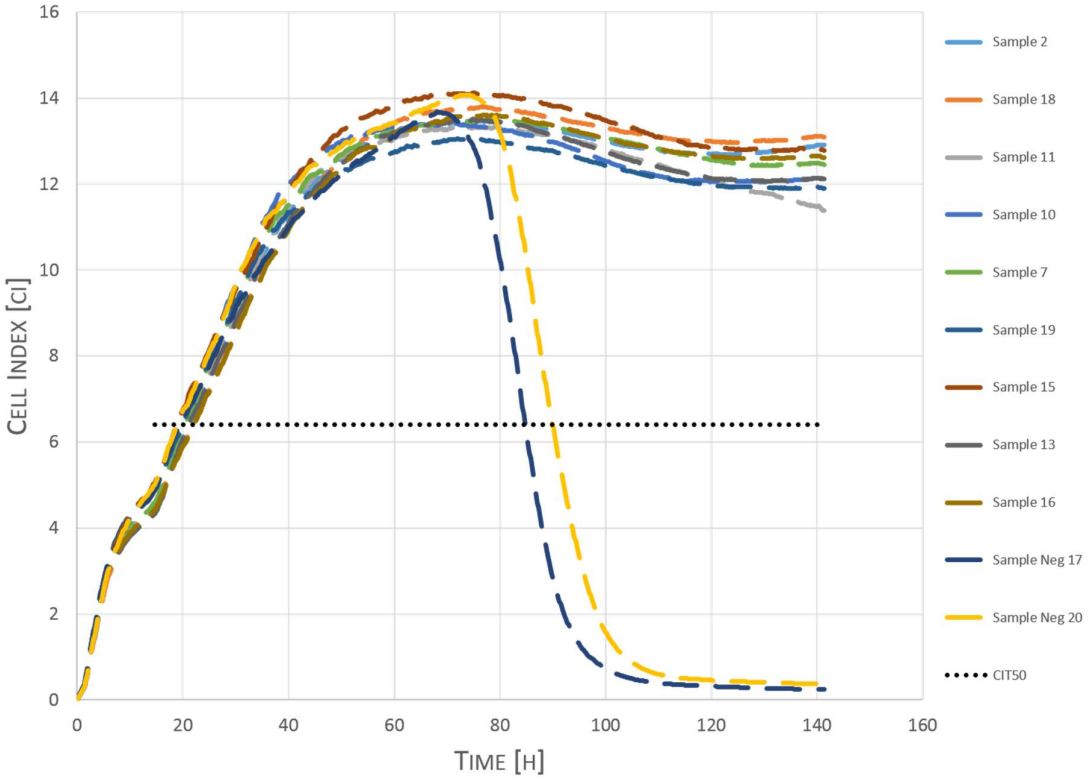


Fig. 60 Neutralizing activity of 11 serum samples
All the serum samples have been diluted 1:8. Sample neg 20 and Sample neg 17 are the only two samples, which show non-neutralizing activity. All the remaining samples show neutralizing activity and hence no substantial decrease in their CI_x value. Therefore the antibody titer values of these samples have been defined as > 1:8

All these serum samples have been 1:8 diluted and showed neutralizing activities as they had minimal decrease in their CI_x values. Therefore, all of these samples had a titer of above one to eight (>1:8) as we couldn't calculate their $RTCAT_{50}$ value because none of these graphs crossed the CIT_{50} value.

Finally, results obtained by the method established by Witkowski et al. [99] were compared to the results by the method established by Fang et al [98]. As the antibody titer of only the first nine samples have been determined by the method of Witkowski et al. [99], results of these samples have been compared. Out of these nine samples, three samples, namely 1, 3 and 6, have been removed from the comparison. The reason was due to the $qRTNT_{50}$ and $RTCAT_{50}$ value respectively, which fell out of the range of two dilutions below or above the corresponding *NT* value. Furthermore, for all samples, which had more than one valid result, mean titer values have been calculated in order to simplify the comparison. The results are shown in the next table (Table 17).

Table 17 Comparison of antibody titers *qRTNT$_{50}$* and *RTCAT$_{50}$* obtained by two different mathematical models

As is evident, only sample 5 obtained titer values, which had a difference of more than two dilutions from each other. Titer values of the remaining samples lay all in the range of one dilution from each other

SAMPLE	NT_{50}	$qRTNT_{50}$	$RTCAT_{50}$
2	1:1024	1:953	1:1623
4	1:32	1:48	1:45
5	1:256	**1:80**	**1:402**
7	1:256	1:401	1:290
8	1:512	1:515	1:861
9	1:128	1:96	1:106

It is notable that all the samples apart from sample 5 show *qRTNT*$_{50}$ and *RTCAT*$_{50}$ values, which have a difference of only one dilution from each other. This looks different for sample 5, where the difference is more than two dilutions apart from each other.

4 Discussion and Outlook

In this chapter, results of the three projects presented in chapter 3 are discussed. This chapter is organized into three sections, each representing the respective project. At the end of each section a brief outlook on further steps for the future is given. The chapter starts with the immunogenicity study of the yellow fever vaccine co-administered with MenAfriVac, then carries on with the establishment of an indirect ELISA for the detection of YF IgG and IgM in serum samples and finally ends with the establishment of virological methods on the xCELLigence™ system as well as the application of two mathematical models for the quantification of neutralizing antibodies against YFV.

4.1 Immunogenicity of the Yellow Fever Vaccine Co-administered with MenAfriVac in healthy infants in Ghana and Mali

In 1991 YF-17D vaccination was incorporated in the Expanded Program of Immunization (EPI) in Africa [73]. Since then a lot more vaccines, like rotavirus, pneumococcal conjugate and human papilloma virus vaccines [100], have been included in the EPI even though only limited data is available on the effect of simultaneous administration of vaccines on the immune response in infants. Therefore, the objective of these studies was to analyze the immunogenicity of the YF-17D vaccine when administered concomitantly with measles and MenAfriVac.

In both studies it could be demonstrated, that PsA-TT (at 10 µg, 5 µg and 2.5 µg dosages) did not adversely affect the immune response to the concomitantly administered YF vaccine at the age of 9 months.

Furthermore, in both studies non-inferiority of each of PsA-TT vaccine group to the control group (YF/Measles vaccines alone) was demonstrated for the majority of pairwise comparisons when viewing the percentages of vaccinees who have achieved seroconversion and seroprotection, four weeks after immunization. In a few instances, such non-inferiority was not confirmed. This could be due to low statistical power, which resulted from low seroconversion rates in study A or from the small sample size in study B. In study A, 68% to 79% of subjects reached YF seroprotection ($NT \geq 1{:}8$) at four weeks after immunization. This is significantly less than the expected 95%, resulting in a low power in testing non-inferiority. In study B, YF endpoints were measured only in a random subsample of subjects (300/1500, 60 subjects per study group), which also resulted in limited power. However, there was no statistically

significant difference among all study groups in each study, in YF virus neutralizing antibody GMTs four weeks after immunization after adjusting age, sex, and pre-vaccination titer.

It could be noticed that there was a major difference between the two studies when looking at the immune response to YF, as measured by *NT* four weeks after immunization. Study B, which was conducted in Mali, had a higher seroconversion rate, seroprotection rate and GMT, respectively, 93%, 97% and 32. In contrast, when we look at study A, which was conducted in Ghana, the results are 68%, 73% and 14, respectively. There are several determinants, which could explain this difference - vaccine sub-strain, vaccine concentration, presence of maternal antibodies and interference of other vaccines [101].

Two different vaccine sub-strains of YF17D were used in the two studies: the 17DD sub-strain in study A (Ghana) and the 17D-213/77 sub-strain in study B (Mali). The 17D-213/77 sub-strain is a derivate of the 17D-204 sub-strain. The difference between these two vaccine sub-strains is the passage level (17D-204:235–240; 17DD: 286–287) [102]. However, when comparing nucleotide sequences of both sub-strains there are only minor differences [103]. Camacho et al. and Nascimento Silva et al. have performed studies, where both vaccine sub-strains, 17DD and 17D-213, were tested for immunogenicity in adults and infants [76, 104]. These studies have shown that there was no significant difference in the immune response between the two. Seroconversion rates were 98% in the study of Camacho et al. and 70% to 88% in the other study of Nascimento Silva et al. However, immunogenicity studies of YF 17D vaccines in infants show that immune responses tend to be lower in infants than in adults with seroconversion rates ranging from 70% to 88.8% [74, 76, 101]. These results are similar to our findings in study A.

Interestingly, Nascimento Silva et al. have also reported a significant difference in seroconversion rates when administering YF 17D alone or simultaneously with MMR (measles, mumps and rubella) - 86.5% versus 69.5% [76]. Similar rates were reported in another study, where 9-11 months old infants received the YF 17D vaccine concomitantly with measles vaccine [101]. They obtained seroconversion rates of 72%. In our case, for both studies all infants received YF 17D simultaneously with the measles vaccine. Hence, the results of study A could support the findings of the studies of Nascimento Silva et al. and the collaborative group for studies with YFV. However, as there was no control group in which infants only received the Measles vaccine, no clear statements concerning this matter can be made.

The difference in immune response between the two studies could also be related to a different number of viral particles in the vaccines. There are indications that the number of viral particles

of one dose has an effect on immunogenicity. A WHO expert committee defined a minimum number of viral particles per dose as 3.0 log10 international units (IU), i.e. ~ equivalent to 3.73 log_{10}PFU. In 2013, the latter concentration was supported by a dose-response study of the YF 17DD vaccine conducted by Martins et al. who demonstrated that this minimal dose, which was established by the expert committee of WHO, was as immunogenic as higher doses with little differences in response rates [102]. The concentrations of the vaccines, which were used for both our studies, were above this concentration (Study A: between 4.34 log_{10} PFU and 4.56 log_{10} PFU; study B: between 4.5 log_{10} PFU and 4.7 log_{10} PFU). However, viral concentrations are determined by titrating the virus on susceptible cells. Most commonly Vero or PS cells are used for this purpose, with titers being higher when performing the titration on Vero cells vs. PS cells (a difference ranging from 0.5 to 1 log_{10}). The method for the determination of concentrations is not published. Although we know, that the titration of the study vaccine of study B was performed on PS cells, we have no information regarding this for the study vaccine of study A. It would be very interesting and valuable to test both the YF 17D study vaccines against each other on the same cell system. With this information, a clear statement regarding different virus concentrations and their effect on immunogenicity of vaccinees and consequently on seroconversion rates could be made.

A currently highly debated topic is the announcement by the WHO Strategic Advisory Group of Experts (SAGE) in 2013, that a single dose of the YF 17D vaccine provides life-long immunity. Thus, revaccination every 10 years, is no longer necessary [105]. Gotuzzo et al have reviewed the efficacy and duration of immunity after YF vaccination in order to assess the need of a booster every 10 years [106]. Their findings indicate that in most studies serconversion rates following YF vaccination were above 90% and that they remained above 75% several years after immunization. Furthermore, they found some indications suggesting that a YF booster would only lead to a minor or short-lived increase in neutralizing antibodies due to pre-existing antibodies from primary vaccination [106]. Hence, they concluded that a YF booster dose would not be needed. However, given the rather low neutralizing GMT of 14 after vaccination in study A, the question may rise whether these titer values are maintained throughout life. Conducting a serosurvey in these infants in 3-5 years would be desirable to evaluate whether titers are maintained or decline with time.

Another important issue, which cannot be neglected, is the presence of maternal antibodies and their role in the immunological response in infants. The median age at vaccination (9 months) and the pre-vaccination titers were similar and consistently low in both studies, with 4.5% and 4.0% of the infants with titers ≥ 1:8 prior to vaccination. However, around 50% of the

mentioned 4.5% and 4.0% did not seroconvert after vaccination. In previous studies, it has been reported that maternal antibodies inhibit vaccination as they neutralize the attenuated virus preventing a specific immune response [107]. Hence, specific timing of the measles vaccination has been set to the age of 6 to 9 months [108]. However, there are no such studies available for YF.

Gender differences in response to YF vaccine have been reported in two different studies with contradictory outcomes. The first study by Monath et al. reported that males had higher responses than females for both 17D manufacturers [109]. In the second study, which was conducted by Pfister et al. [110] similar results were shown at first sight. However, for one YF 17D vaccine (Aventis Pasteur) it was observed that response rates in females were higher than in males [110]. The studies of Monath et al. and Pfister et al. were performed in adults, whereas our studies were performed in infants. This difference can be of importance, as the immune system of infants is not fully developed and hence, reacts differently from that of adults. More studies are required to analyze gender differences in immune responses to the YF 17D vaccine in infants.

In conclusion, it can be said that concomitant administration of the PsA-TT does not affect the response to YF vaccine in African infants. Differences in the post-vaccination seroconversion and seroprotection rates in the two studies were observed. This variability in the results can also be noticed in other studies confirming the urgent need to further document the immune response to YF 17D vaccine in infants. This would help in identifying and understanding determinants, which can have an influence on the immune response in infants.

4.2 Establishment of an indirect enzyme-linked immunosorbent assay (ELISA) for the detection of YF IgG and IgM antibodies in serum samples of vaccinees

With the re-emergence of YF in endemic regions the need for large scale vaccination campaigns in order to stop the disease from spreading is of immense importance. Currently, one of the main laboratory assays for identifying outbreaks are based on the serological analysis of patient samples. Till date the most specific test in the serological analysis is the neutralization assay. However, this assay can only be performed by highly trained personnel, needs special laboratory facilities and is time-consuming (it can take up to seven days till the result). Alternatively, an enzyme-linked immunosorbent assay (ELISA) can be performed for the detection of YFV antibodies in serum samples. This assay is not as time consuming and

laborious as the microneutralization assay and would allow the analysis and evaluation of multiple serum samples within one day. Having said that, till date there are no commercial ELISA kits available for the detection YFV antibodies. Therefore, this project is aimed at standardizing and validating an in-house antigen based indirect ELISA for the detection of YF IgG and IgM antibodies in patient samples. Once established, this assay would allow the analysis and evaluation of multiple serum samples within one day.

We were able to establish a protocol for an indirect IgG as well as IgM ELISA, which could detect positive and negative serum samples that were well-characterized by IFA before.

It could be shown that for both the ELISAs the recombinant NS1 protein gave the best result when compared to the other antigens. Furthermore, titration assays of this antigen indicated that a concentration of 1µg diluted with carbonate buffer was the best concentration for this protocol. Looking for the best combination of antigen to serum sample dilution it could be seen that a 1:50 serum dilution worked well. The problem with higher serum dilutions was that these were in the same range as the serum control making a differentiation between unspecific and specific signals difficult.

With the determination of these factors the initial steps for establishing a protocol for an indirect IgG as well as IgM ELISA were completed.

An important key factor in the development of a successful ELISA assay is the specific binding of antigen and antibodies. Therefore, we wanted to use highly immunogenic antigens. The use of recombinant viral proteins as antigens in ELISAs is very common. Studies have shown that the highly conserved Flavivirus prM, E and NS1 proteins are the major targets of the antibody response in humans and animals [111]. Besides these, the non-structural proteins NS3 and NS5 have also shown to be immunogenic [112]. In our case, we have decided to analyze native antigens, from virus infected cell culture supernatants (Asibi and YFV 17D), recombinant E and NS1 protein and also the E-III domain of the E protein.

Surprisingly, in our case only recombinant NS1 showed a strong signal. In the case of the IgG ELISA the native antigens from virus infected (Asibi and YFV 17D) culture supernatant looked promising in the beginning but when compared to the recombinant NS1 protein could not give the same results. For the IgM ELISA, none of these native antigens gave strong signals. Similar problems regarding the intensity of the signals could be seen with the recombinant peptides of the envelope-III domain (E-III domain in Fig. 34 and E3 pp40, E3 pet 44 Fig. 39) as well as the full length envelope protein (E-protein in Fig. 34 and E-full 4T1 in Fig. 39). Furthermore, the difference between the signals of the negative and the positive sample was so small, that, when

deducting the signal of the negative sample from the positive one the resulting signal was very low.

The E-protein is the major surface protein mediating binding of virions to target surfaces and fusion with the membrane. This glycoprotein can be divided into three domains - a ß-barrel structure domain, a dimerization domain and lastly an immunoglobulin-like domain. This last domain shows most of the antigen epitopes, which determine the strain and the type of the virus. Hence, the majority of neutralizing antibodies are directed against these epitopes [112]. It is assumed that this domain is also responsible for receptor binding. Due to these reasons, units of the E-III domain have been considered as target for this ELISA assay. However, these small recombinant subunits could not give the anticipated results when compared to the other antigens. A recent study by David W. C. Beasley [113], where also E-III subunits have been investigated as antigens for the detection of antibodies, has revealed that these subunits can only be poorly bound by the detection antibody. This is largely due to the small size of the fragments and the thus resulting steric hindrance. In our case, we could notice low absorbance values, which could have been caused by this phenomenon. Furthermore, recent literature indicates that domain II of the E-protein of WNV contains a strong immunodominant epitope, against which most of the antibodies are directed [112, 114]. This is in contrast to prior publications, in which most of the epitopes have been described to be located in domain III of the E protein. Additionally, studies have shown that Flavivirus E protein undergoes a series of different conformations. Therefore, it has been suggested that most of the target epitopes are buried in the protein, thus preventing the binding of antibodies. This could have been the cause for the low absorbance values of the full-length E-protein in our case [17]. Also, it has been reported that most of the cross-reactive epitopes are located on the E protein, which are the cause for the serological cross-reactivity amongst Flaviviruses [115]. This could explain the high absorbance values of the negative control when the E protein was used as an antigen.

NS1 is a glycoprotein, which is highly conserved in the Flavivirus genus, existing in intracellular, cell surface and secreted forms [36, 37]. The exact functions of this protein are still not fully clear. However, it is reported that the intracellular dimer NS1 is a key factor in genome replication and the secreted hexamer plays an essential role in immune evasion.

In contrast to the E protein, the NS1 protein is thought to be less cross-reactive as more species-specific epitopes can be found on this protein. This is in accordance to our findings as we obtained the best OD values with this antigen for the IgG as well as IgM ELISA.

The remaining antigens used for this assay were the supernatants of Asibi and YFV-17D infected cells. In the case of the IgG ELISA both the antigens looked promising in the beginning. However, when compared to NS1 could not give same results. Whole virus antigens lead to false positive results as they cross-react with antibodies against other Flaviviruses as well as other unrelated infectious agents [116]. This is reflected in the high OD values of these antigens for positive as well as negative serum samples in our case. The μ-technology ELISA, which was tested for the IgM detection showed similar results – high OD values for positive as well as negative serum samples. This again is an indication for high cross reactivity with other Flaviviruses or rather antigens of these and other unrelated infectious agents as already mentioned above.

The assay was initially evaluated with well-characterized serum samples and yielded no false positive results. It is necessary to further deepen the analysis in regard to detection limits of the assay with more negative samples. Furthermore, specificity of the assay should be analyzed with samples from other Flavivirus infections. The protocol also offers scope for improvement in regard to antigen concentration and serum dilution. Extensive testing of this antigen would help to establish parameters for clear discrimination of positive, negative, and equivocal results.

Nevertheless, we could demonstrate that both the IgG and the IgM ELISA worked well with NS1 as antigen and that the results of the initial evaluation were significant enough. In order to further test and evaluate this assay, serum samples of study B were analyzed for IgG and IgM antibodies as well. All of these samples were assayed in a microneutralization assay in regard to neutralizing activity against the YFV and hence had *NT* values.

A total of 187 YFV negative serum samples and 190 YFV positive samples were analyzed. All these serum samples were taken from infants about nine months of age four weeks after yellow fever vaccination. The GMT of the YFV positive samples was 32, which lies in the middle range of titer values and a total of 97% of analyzed subjects reached seroprotection (*NT* above 1:8). The 190 subset of serum samples tested here had different titer values ranging from 1:8 to 1:128. The pool of negative samples was used to determine the cut-off value for each assay, i.e. IgG ELISA and IgM ELISA respectively. The cut-off for the IgG ELISA was calculated to be 0.963 and for the IgM ELISA 0.424.

Our analysis of the samples has shown that 57 (30%) YF-17D vaccinees were IgM positive and 143 (75%) were IgG positive. In contrast, 133 (70%) of the YF-17D vaccinees were IgM negative and 47 (25%) were IgG negative. Furthermore, deeper data analysis of these samples has revealed that out of the 133 (70%) IgM negative vaccinees, 91 (48% of the total of 190

samples) were IgG positive and 42 (22% of the total of 190 samples) were IgG negative. Similarly, 47 (25%) of the IgG negative vaccinees had 5 (3% of the total of 190 samples) IgM positives and 42 (22% of the total of 190 samples) IgM negatives. This means that a total of 42 (22%) samples were completely negative for IgM as well as IgG antibodies.

Typically, antigen specific IgM antibodies appear by day seven upon vaccination, peak at about two weeks afterwards and slowly decline thereafter. The duration of IgM antibodies detectable in patient samples varies very strongly from patient to patient and hence is unknown. At the end of the first week of illness YF-specific IgG antibodies can be detected in low concentrations. However, these concentration levels increase steadily thereafter and have shown to persist for 30 to 35 years after a single vaccination [82, 117-119]. Therefore, high levels of IgM antibodies are an indication of a recent infection/immunization with the virus whereas high levels of IgG antibodies indicate a past infection/immunization.

Interestingly, the fact that out of the 133 IgM negative samples, 91 (68% of a total of 133 samples) were IgG positive and that 5 out of the 47 IgG negative samples were IgM positive (i.e. 11% of a total of 47 samples) is in accordance with the current state of knowledge about the development of antibodies after an infection and vaccination, respectively. Hence, it is safe to assume that those samples, where IgM couldn't be detected, were initially IgM positive. Similarly, this applies to the IgG negative samples, where 11% were IgM positive. It can be assumed that if tested at a later time, it is likely to detect IgG antibodies.

In this analysis, samples were used, which were definitely positive for neutralizing antibodies against YFV. Therefore, IgG or IgM antibodies should have been detected in all of these 190 samples. However, it could be seen that in 42 samples, i.e. 22% of the total of 190 samples, neither IgG nor IgM could be detected. The detection system used in this assay is based on the antigen NS1. Therefore, only NS1-specific IgG and IgM antibodies can be detected. One possibility, which could explain the 22% negative samples, is that other antigen specific antibodies were not detected. The weak reactivity of the sera could be also caused by the presence of anti-prM/E IgG/IgM antibodies in the sample. For other Flaviviruses it could be seen, that these antibodies can hamper anti-NS1 IgG/IgM antibodies from binding to the antigen thus resulting in low detection levels. Studies have shown, that a single incubation step of serum samples with prM and E antigens can deplete the serum of these antibodies and hence increase the detection of anti-NS1 IgG/IgM antibodies [120-122]. For the IgM detection, these samples have been incubated with an RF absorbent to remove IgG antibodies and rheumatic factors from the serum. It would be interesting to include the incubation step with the above-mentioned

antigens to see if this improves detection levels. Furthermore, one point which should not be neglected in this part are the effect of the serum quality on the assay or rather assay result. All the samples used for both the assays had been stored at -20°C after the usage in the microneutralization assay. Therefore, they had to be thawed prior usage for these assays. Studies have shown that the concentration of antibodies in the serum decreases with time as well as with the amount of freeze and thaw cycles [123]. Hence, multiple thaw and freeze cycles of these samples could have had an effect on the antibodies resulting in very low levels, which could not be detected.

Comparison analyses of OD values and respective *NT* values have demonstrated that there is no correlation between these two for IgG as well as IgM samples. Low *NT* values did not necessarily mean low OD values and vice versa. The reason for this could lie in the distinct nature of the two assays. The neutralization assay is a functional assay, which detects neutralizing activities induced by IgG and IgM antibodies. In contrast, the ELISA measures IgG or IgM antibodies, which are bound to the coated antigen. Therefore, it is questionable if these two values are comparable at all.

Most laboratories use ELISA assays, which have been established and validated in-house as there are no commercial anti-YF IgG/IgM ELISA kits available. Till date the use of a similar assay as established here in this thesis has not been reported as being commercially available. Generally, a number of ELISA assays against dengue, WNV and JEV have been published in recent years [121, 124-126]. However, the detection methodologies of these assays are different to our detection methodology. Currently, the guidelines of the WHO include a basic ELISA test for the confirmation of YF IgM/IgG antibodies in patient samples prior to a neutralization assay [127]. This YF IgM/IgG capture ELISA has been successfully developed and published by the Center for Disease Control and Prevention (CDC) in the USA [128, 129]. Nevertheless, the difference to the ELISA established here is that our uses an antigen whereas CDC's is based on the use of anti-human IgM/IgG antibodies. As the CDC ELISA is included in the laboratory testing algorithm of the WHO for the confirmation of a YF case, most reference laboratories in Africa use this assay. However, to date no data has been published, where the presence of neutralizing antibodies has been correlated to IgG and IgM antibodies detected by an immunoenzymatic assay. Such an efficacy trial as performed here with serum samples tested by a microneutralization assay as well as an indirect ELISA would be helpful in further evaluating the indirect ELISA, which was established here in this thesis.

Concluding, it can be said that this assay still yielded a total of 78% positive samples (IgG and IgM), making it a useful tool for the analysis of serological parameters. In the future, it would be worthwhile to test other antigens, especially recombinantly fused antigens of the E, prM and NS1 protein to increase sensitivity of the assay. Compared to the robust microneutralization assay this assay is not as laborious as it can be quickly performed using only little equipment. Furthermore, execution and performance of the assay requires only minimal training, saving overall time and cost.

4.3 The xCELLigence™ system – an alternative tool to classical virological methods

The aim of this project was to transfer and establish standard virological methods using the xCELLigence™ system in order to simplify the execution of experiments and more importantly the subsequent evaluation. Thereby two distinct mathematical models from two different publications [98, 99] have been employed. The results of these and the ones obtained by the established microplate assays have been compared.

The xCELLigence™ system is a novel tool developed by Roche Applied Science in collaboration with ACEA Biosciences. This system measures impedance changes resulting from cell attachment and interaction with the microelectrodes on the bottom of wells on a 96 E-Plate in real time. These changes are displayed as cell index values, which correlate with cell number, size and shape.

To begin with a viral titration assay was performed on the xCELLigence ™ system. For the determination of the virus titer of four different virus stocks A, B, C and D, a virus solution of known titer was used as standard. The determination of the virus titer was done with the mathematical model set up by Witkowski et al. [99]. We were able to show that when comparing results of this model with those of the standard microtitration assay, both the assays produced almost identical results. Therefore, the results of the assay on the xCELLigence™ system were considered validated.

However, there was one noticeable weak point in the determination strategy of the virus titer. A specific threshold has to be set, which varies from experiment to experiment. The requirement for this threshold is that it should cover a broad range of virus concentrations, which are used as standard. It would be interesting to compare virus titer values of one experiment obtained by setting different thresholds in order to determine the assay to assay variation.

The next step was to perform a neutralization assay. The protocol used for this system was similar to the standard microneutralization assay. The mathematical model for determining antibody titers reflecting 50% neutralization of the virus, the so called $qRTNT_{50}$, was also established by Witkowski et al [99]. We were able to determine the neutralization titers of 9 serum samples. These samples have already been analyzed by the standard microneutralization assay and hence had *NT* values as well. Unlike the *NT* values, the $qRTNT_{50}$ can have any value and not be restricted to stringent two-fold dilutions. Therefore, in order to evaluate $qRTNT_{50}$ values obtained by this method, dilutions lying above or below corresponding *NT* values were compared. It could be seen that four samples out of nine (sample 2, 3, 7 and 9), i.e. 36% - showed a difference of one dilution between the new value and the corresponding *NT* value. Two samples (sample 4 and sample 8), i.e. 18% - had no variances in their titer values when compared to the corresponding *NT* values. In contrast two other samples (sample 1 and sample 6), i.e. also 18% - had a difference of more than two dilutions between the $qRTNT_{50}$ and the *NT* value. The question now is whether a variance of one dilution between two methods is acceptable. The assay is based on the interaction of different biological components as cells, virus and substances of the serum sample. Hence, these components can interact differently from experiment to experiment. Therefore, a certain assay to assay variability can be assumed. Furthermore, the classical neutralization assay as well as the microneutralization assay has a tolerated fluctuation of one and two dilutions respectively. Hence, a variance of one dilution is acceptable. However, when looking at samples, which have titer values near the cut-off of 1:8, a stricter evaluation has to be performed in order to exclude false negative and false positive results, respectively. In these cases, there should not be any variances between the dilutions at all. When looking at the assay to assay variation, it could be seen that two samples (sample 2 and 7) had higher variations of two dilutions when compared to each other. Only one sample (sample 4) showed a difference of one dilution and another one (sample 3) a difference of more than two dilutions. As already mentioned above these variations are acceptable as we are working with biological substances. However, the same exceptions have to be made when analyzing weak positive samples. Using this method, the same weak points became evident as mentioned above. For the determination of the $qRTNT_{50}$ a time point of determination is set, where the time point chosen for the calculation of $qRTNT_{50}$ is not defined. Questionable hereby is the fact that the result of one sample would vary dependent on the chosen time point. One solution for this problem could be to set a definite time point as a guide value and to then compare the serum titer of several experimental runs with different serum samples but always at the same time point with results of the microneutralization assay. The time point of

determination gives too much room for variability in the results, making this an issue for further investigation.

Following the above, another strategy for the determination of neutralizing antibody titers against YFV in serum samples has been presented in this thesis. This mathematical model was established by Ying Fang [98]. The approach here was a bit different than before as the method involved the use of serum standards with known antibody titers. We were able to establish different serum standards, with which neutralizing antibody titers of serum samples could be calculated. In this way, a total of 20 serum samples could be analyzed in regard to their neutralizing activities. As different standards have been used for the determination of neutralizing antibody titers of same samples, some of these had multiple results. Therefore, each result, i.e. the $RTCAT_{50}$ was compared to its corresponding result of the standard microneutralization assay, the *NT* value. The $RTCAT_{50}$ value as well can have any number and is not restricted to stringent two-fold dilutions. The comparison of the two results has shown that 3 out of 20 samples (sample 1, 2 and 3), i.e. 15% - have one $RTCAT_{50}$ value each, which lay above or below the range of two dilutions from the corresponding *NT* values. However, two samples (sample 1 and sample 2) had multiple results, which again lay in the range of two dilutions above or below the corresponding *NT* value. It is evident that 5 samples have a difference of one dilution between the *NT* and $RTCAT_{50}$ value. The remaining samples have a difference of two dilutions. The same question arises as with the first method presented in this part of the thesis. Is a variance of two dilutions acceptable? Here the same response applies as above. The assay is based on the interaction of different biological components as cells, virus and substances of the serum sample. As all of these components are of biological nature, the interaction of these can differ from experiment to experiment leading to the variations in neutralizing titer values. Therefore, we have permitted a range of variation of two dilutions in our standard microneutralization assay. As the method is identical using the same components this acceptability criteria can be applied here as well. However, as also mentioned above, this can become critical when analyzing weak positive samples. Here the acceptance criteria have to be stricter – which means allowing no variation in their titer values in order to exclude false positive and false negative samples.

Generally, when looking at the method of determination of the $RTCAT_{50}$ value itself, some weak points became evident. This method used serum standards with known neutralizing antibody titers. The weak point hereby was to obtain proliferation curves, where every serum dilution would show a decrease in their respective CI_x value and where each dilution would have the same distance to each other. While establishing serum standards for this method we had a few

samples, where some serum dilutions were not equidistant to each other. This resulted in linear regression curves with a poor correlation coefficient R^2 of below 0.95 indicating that there was not a clear linear relationship between antibody titer and time of decrease at CIT_{50}. Hence, results in the form of antibody titers which have been calculated by the linear regression equation were not reliable anymore. Another difficulty resulting from these kinds of graphs is that only a small range of antibody titer values can be covered if some of the dilutions do not show any decrease in the CI_x value and hence have no intersection point with the CIT_{50} value for the given period of time for this experiment (i.e. 140 to 160 hours). Therefore, calculated neutralizing antibody titers of samples tested in these runs lay outside of the neutralizing antibody titer range of the standard.

Another problem we encountered while establishing the standards, was that each run of the same serum standard gave different proliferation curves. This again resulted in different neutralizing antibody titers for the same sample. An approach here could be to determine time boundaries in which samples are definite positive or negative. These boundaries can be determined with the help of the negative serum sample. The time point of decrease in the CI_x value of the negative serum sample can be the boundary of negativity. Hence, samples where the CI_x value decrease at the same time, are counted as negative. For the determination of such a time boundary a set of negative serums samples should be tested in this system and the mean time point of decrease in their respective CI_x values should be determined as cut off value for this system. As we are looking at time points where each dilution curve declines, it would make more sense to determine a time point as cut off value than a dilution for this system. This could be especially crucial when analyzing weak positive samples.

The use of the correct dilution of the serum sample to be analyzed was another difficulty. As shown in Fig. 60 some of the samples we have used and which had been diluted 1:8 did not show any decrease in their CI_x value. Therefore, respective neutralizing antibody titers could not be calculated as the curve did not intersect with the respective CIT_{50} value at the given time period of respective experiment. A solution here would be to strongly dilute all samples before use and then to consider the dilution factor when calculating the neutralizing antibody titer.

Comparing the results of both methods it could be seen that the differences in neutralizing titer values between them were of only one dilution for all samples except one. However, when looking at the determination strategies of neutralizing antibodies the method established by Witkowski et al. posed fewer difficulties than the method of Fang et al. The major problem with the latter method was establishing a standard, which could be used for the calculation of the

samples. A good alternative to using serum samples as standard could be the use of commercially obtained monoclonal antibodies (mABs) against YFV as these would be free of interacting substances and hence could give well defined cell proliferation graphs of each mAB concentration. Generally using serum samples as standard poses a lot of problems regarding storage. Studies have shown that with time and also amount of freeze and thaw cycles the concentration of antibodies decreases as they are not so stable. Even storing serum samples at 4°C in the fridge shows a decrease in the antibody titer after 14 days [123]. Therefore, special care has to be taken in the handling. The standards used for this project have been already tested in various other projects and therefore had unknown freeze and thaw cycles. However, before using these serum samples for this project all of them have been aliquoted in appropriate dilutions and volumes so that for each experiment a new tube of the same sample could be used. Nonetheless, serum sample material is sparse. Hence, it is not possible to store loads of aliquots of the same sample in an appropriate dilution. Therefore, a very good alternative would be a mAB against YFV. The establishment of this could be the focus for a future study.

Concluding this project, it can be said that the xCELLigence™ system can surely serve as a tool for virological methods, as viral titration as well as virus neutralization worked well on this system. However, now that a preliminary protocol has been established, it is necessary to carry out further testing with many more samples and to compare the obtained results to the classical methods in order to exclude potential nonconformities. Especially the problem with the low positive samples should be taken into focus as this would be crucial for the reliable use of this system. Once established the method of Fang et al. holds a lot of promise due to the one-well detection system. This would be especially useful in big immunogenicity studies, where multiple serum samples have to be tested in regard to their neutralizing activity.

Both methods have shown, that the xCELLigence™ system allows a very fast qualitative statement of the analyzed serum samples. Hence, for screening serum samples in regard to their neutralizing activity without quantitation purposes this serves as a very good tool.

One critical point is the question of the cost-benefit ratio. Especially when looking at the time factor it could be seen that the experiments for viral titration as well as virus neutralization have taken a minimum of 5 days. This is the same time classical titration or neutralization assays need before they can be evaluated. However, the tedious work of fixing cells, staining them and evaluating each well under the microscope is not required. Furthermore, this system allows normalized real-time measurement of cell proliferation and viability, without subjectivity, and also is computerized. This saves time and makes the evaluation more accurate.

All in all, the xCELLigence™ system appears to have great sensitivity for detecting changes in infected cells that may not necessarily be observable under conventional microscopy. Therefore, it may be worthwhile to use this system for monitoring non-cytopathic virus infections where small or subtle changes in the cell monolayer may be detected. Furthermore, this system can be used for studying and evaluating antiviral vaccines, screening for effective antiviral agents, screening samples for the presence of viral infections and neutralizing antibodies without quantitation purposes. The method is objective, automated, allows for a high-throughput of samples and for the continuous collection of data in real-time.

Appendix: Materials

A.1 Laboratory Equipment and materials

Laboratory Equipment

Equipment	Supplier
Autoclave • Varioklav • Automat 21/2	H+P Labortechnik GmbH (Oberschleißheim) Webeco (Bad Schwartau)
Clean bench • Clean bench small • Clean bench	The Baker company Thermo scientific
Centrifuge • Sigma 3K30C • Sigma 1-13 • Sigma 1-40 • Sigma 1-15K	Braun Biotech International (Melsungen)
ELISA-Reader Infinite®200	Tecan Group Ltd. (Männedorf, Switzerland)
Freezer • -20°C • -80°C • -80°C	Liebherr (Biberach a.d. Riss) Sanyo (Japan) Heraeus (Hanau)
Gel documentation system	Peqlab (Erlangen)
Gel electrophoresis Systems	Biozym (Oldendorf) Bio-Rad (Hercules, USA)
Glassware	Schottglas (Mainz)
Incubator • Cell culture (37°C with 5% CO_2) • Cell culture (37°C without 5% CO_2) • Bacterial culture	Heraeus (Hanau) Heraeus (Hanau) New Brunswick scientific (USA)
Microscope • Optical light microscope Telaval 3 • Optical light microscope Motic AE21	Zeiss (Jena)
Microwave	Bosch (Stuttgart)
NanoDrop™ ND 1000	Peqlab (Erlangen)
Neubauer - Cell counting chamber	Roth (Karlsruhe)
Photometer (Biophotometer)	Eppendorf (Hamburg)
Pipette • 2µL, 2-10µl, 10-100µl, 100-1000µl	Eppendorf (Hamburg)
Rotors • SW 32 Ti • Typ19	Beckman (Palo Alto, USA)
Sequencer (ABI PRISM® 3100 genetic analyzer)	Applied Biosystems (USA)

Scales • B310 P (agarose gel) • L610 D	Sartorius (Göttingen)
Thermocycler • Biometra T gradient cycler	Biometra (Göttingen)
Thermoblock • Thermomixer compact • Thermomixer comfort	Eppendorf (Hamburg)
Transilluminator TC-312A	Spectroline (Westburry, USA)
Trans-Blot® Semi-Dry Electrophoretic Transfer Cell	Bio-Rad (Hercules, USA)
Vortex®	Roth (Karlsruhe)
Voltmeter	Biometra (Göttingen) Bio-Rad (Hercules, USA)
X-raying film development system Curix 60	Agfa-Gevaert Group (Mortsel, Belgium)
xCELLigence™ System	Roche/ACEA Biosciences Inc. (Mannheim)

Materials

Type	**Supplier**
ABgene PCR plattes (Thermo fast 96)	Abgene (Epsom, UK)
Cell culture flask (25 cm^3, 75 cm^3, 175 cm^3)	Nunc (Wiesbaden)
Cell scrapers • 24cm length, 13mm blade width • 38cm length, 25mm blade width	Peske Laborbedarf (Aindlingen-Arnhofen) Peske Laborbedarf (Aindlingen-Arnhofen)
Cryo tubes (1.8 mL)	Nunc (Wiesbaden)
Development cassette for X-ray	Appligene (Heidelberg)
Disposable Seropipette (2mL, 5mL, 10 mL, 25mL)	Braun Biotech International (Melsungen)
Eppendorf tubes (0.5 mL, 1.5mL, 2mL)	Eppendorf (Hamburg)
Falcon tubes (15mL, 50mL)	Nunc (Wiesbaden)
Glassware	Schott Glas (Mainz)
Glass slides for IFA	EUROIMMUN AG (Lübeck)
Immobilon®-P Transfer Membrane (PVDF)	Millipore/Merck (Darmstadt)
Parafilm	Pechiney Plastic Packaging (Chicago, USA)
PCR 8 strip tubes	Eppendorf (Hamburg)
PCR Plates with 96 wells	Abgene (Epsom, UK)
Petri dishes	Greiner Bio One GmbH (Frickenhausen)
Pipette tips with filter (10µL, 100µL, 1000µL)	Biozym (Oldenburg)
Pipette tips without filter (10µL, 100µL, 1000µL)	Eppendorf (Hamburg)
Quartz Cuvette	Eppendorf (Hamburg)

Ready-to-Use gels - 12 wells (Western blot) 8-16% Precise™ Tris-Glycine 12%	Thermo Fisher Scientific Inc. (Waltham, MA, USA)
Scalpel (disposable)	Braun Biotech International (Melsungen)
Titerplane	Euroimmun AG (Lübeck)
Ultra-clear cap strips	Abgene (Epsom, UK)
Well-Plate (6-well, 24-well, 96-well)	Nunc (Wiesbaden)
Whatman Blotting paper (Filter paper for Western blot)	Schleicher & Schuell (Dassel)
X-ray film CL-XPosure™	Thermo Fisher Scientific Inc. (Waltham, MA, USA)
YFV Microtiter Plates (coated with anti-human IgM)	NovaTec (Dietzenbach)

A.2 Chemicals

Chemical	Supplier
Acetone	Roth (Karlsruhe)
Agar	Invitrogen (Karlsruhe)
Agarose (NuSieve®3:1)	Biozym (Oldenburg)
Amido Black	Roth (Karlsruhe)
Ammonium peroxodisulfate (APS)	Roth (Karlsruhe)
Ampicillin	Sigma-Aldrich (München)
ß-Mercaptoethanol	Sigma-Aldrich (München)
Bovine Serum Albumin (BSA)	New England Biolabs (Frankfurt a. Main)
Bromphenolblue	Sigma-Aldrich (München)
Calcium Chloride	Roth (Karlsruhe)
Carboxymethylcellulose (CMC)	BDH Chemicals Ltd. (Poole, England)
Chloramphenicol	Roth (Karlsruhe)
Chloroform	Roth (Karlsruhe)
Dimethyl sulfoxide (DMSO)	Merck (Darmstadt)
Desoxyribonucleosidtriphosphat-Mix (dNTP-Mix) [10mM]	Eppendorf (Hamburg)
Dithiothreitol (DTT) [0.1M]	Invitrogen (Karlsruhe)
Ethanol	Roth (Karlsruhe)
Ethidiumbromid	Roth (Karlsruhe)
Ethylenediaminetetraacetic acid (EDTA)	Merck (Darmstadt)
Evan's blue	Sigma-Aldrich (München)
Fetal bovine serum (FCS)	PAA Laboratories (Linz)
Formaldehyde	Roth (Karlsruhe)
Glutamine	Roth (Karlsruhe)
Glucose	Roth (Karlsruhe)
Glycerol	Roth (Karlsruhe)
Guanidinthiocyanat (GTC)	Roth (Karlsruhe)
IIFT-mounting medium	EUROIMMUN AG (Lübeck)
Isopropanol	Merck (Darmstadt)
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	Roth (Karlsruhe)
Lipofectamin™ 2000	Invitrogen (Karlsruhe)

Magnesium acetate [280 mM]	TwistDx Limited (Cambridge)
Magnesium chloride ($MgCl_2$) [50 mM]	Invitrogen (Karlsruhe)
Methanol	Roth (Karlsruhe)
Mono sodium phosphate (NaH_2PO_3)	Merck (Darmstadt)
Penicillin/Streptomycin [100.000 U]	PAA Laboratories (Linz)
(Roti®-) Phenol	Roth (Karlsruhe)
Polyacrylamide (PAA) (Rotiphorese® Gel 30; 37.5:1)	Roth (Karlsruhe)
Poly-L-Lysin	Sigma-Aldrich (München)
Potassium chloride (KCI)	Merck (Darmstadt)
potassium dihydrogen phosphate (KH_2PO_3)	Merck (Darmstadt)
ROX Reference dye	TIB MolBio (Berlin)
Saccharose/Sucrose	Sigma-Aldrich (München)
Sodium chloride (NaCl)	Merck (Darmstadt)
Sodium bicarbonate ($NaHCO_3$)	Merck (Darmstadt)
Sodiumdodecylsulfate (SDS)	Serva (Heidelberg)
Sulphuric acid	Merck (Darmstadt)
N, N, N', N'-Tetramethylethylenediamine (TEMED)	Roth (Karlsruhe)
Tris-Hydroxymethyl-Aminomethane (Tris)	Roth (Karlsruhe)
Tris-Hydroxymethyl-Aminomethanehydrochloride (Tris-HCL)	Sigma-Aldrich (München)
Triton X-100	Sigma-Aldrich (München)
Tryptose-Phosphate-Broth	Sigma-Aldrich (München)
Tween® 20	Merck (Darmstadt)
Water (RNAse free for molecular use)	Eppendorf (Hamburg)

A.3 Buffer and other chemical solutions

Name	Ingredients	Supplier
5x-Buffer (PCR)	-	Invitrogen (Karlsruhe)
10x-Buffer (PCR)	-	Invitrogen (Karlsruhe)
10x Ligation Buffer	-	New England Biolabs® Inc. (Ipswich, USA)
BugBuster® Protein Extraction Reagent	-	Merck (Darmstadt)
Buffer for restriction enzymes (10x)	-	New England Biolabs® Inc. (Ipswich, USA)
Lane Marker Sample Buffer (5x) (Western Blot)	-	Thermo Fisher Scientific Inc. (Waltham, MA, USA)
Carbonate Buffer (ELISA)	-	NovaTec (Dietzenbach)
Citrate Buffer (ELISA)	-	NovaTec (Dietzenbach)
Coomassie	2.5 g/l Coomassie 450 mL Methanol 100 mL acetic acid ad 1 L aqua dest.	-
Destainer Coomassie	200 mL Ethanol 50 mL acetic acid 250 mL aqua dest.	-
Diluent solution	8 g/L NaCl 0.4 g/L KCl 0.06 g/L Na_2HPO_4 0.06 g/L KH_2PO_4	-

	1 g/L glucose 0.37 g/L $NaHCO_3$ adjust to pH 7.0 add 0.2% EDTA	
DNA Loading Buffer (6x)	5 mL Glycerin 40 mg Bromphenolblue 5 mL TBE	-
Naphthalene black solution	1 g naphthol blue black 13.6 g sodium acetate 60 mL glacial acetic acid ad 1 L aqua dest.	-
IgG Sample Diluent (ELISA)	-	NovaTec (Dietzenbach)
IgM Sample Diluent (ELISA)	-	NovaTec (Dietzenbach)
Formaldehyde (3.7%)	100 mL Formaldehyde (37%) 100 mL 1 x PBS 800 mL aqua dest.	-
PBS (phosphate-buffered saline) buffer	140 mM Nacl 2 mM KCl 10 mM Na_2HPO_4 2 mM KH_2PO_4	-
Ponceau S (0.1% (w/v) Ponceau S in 5% (v/v) acetic acid)	1 g Ponceaus S 50 mL acetic acid ad 1 L aqua dest.	-
SAP (shrimp alkaline phosphatase) Buffer (10x)		New England Biolabs® Inc. (Ipswich, USA)
SDS 10%	10g SDS 100 mL aqua dest.	-
SDS Sample Buffer (4x) (Western Blot) (with or without ß-Mercaptoethanol)	50 mM Tris; pH 6.8 40% Glycerine 8% ß-Mercaptoethanol 4 g/l Bromphenolblue 80 g/l SDS	-
SDS Running Buffer TGL (5x) (Western Blot)	15.1 g/l Tris 94 g/l Glycine 50 mL SDS (10%)	-
Stop Solution (ELISA)	-	NovaTec (Dietzenbach)
TBE (1x, pH8)	10.8g Tris Base 5.5g boric acid 0.7g EDTA-Na_2	-
TBST (10x)	250 mM tris 1.5 M NaCl 0.5 % Tween-20	-
TMB Substrate	-	NovaTec (Dietzenbach)
Transfer Buffer (Western Blot)	25mM Tris 150 mM Glycine 10% SDS 20% Methanol	-
Washing Solution 20x (ELISA)	-	NovaTec (Dietzenbach)
Western Blocking Reagent		Roche/ACEA Biosciences Inc. (Mannheim)

A.4 Media

Media for cell culture

Medium	Additional	Supplier
DMEM (Dulbecco's Modified Eagle Medium)	10% FCS, 1% Penicillin-Streptomycin, 1% L-Glutamin	GIBCO Invitrogen (Karlsruhe)
L15 (Leibovitz-Medium)	5% FCS, 1% Penicillin-Streptomycin, 1% L-Glutamin	GIBCO Invitrogen (Karlsruhe)

The media contain anorganic salts, amino acids, vitamins and other components additionally. Furthermore, Phenol red is added as a pH indicator, changing its color from red to yellow when the medium becomes too acidic (from pH 7.4 to pH 7.0). Hence, this is an indication for the consumption of the medium.

Other media

Medium	Additional	Supplier
S.O.C. medium (super-optimal catabolite repression broth)	-	Invitrogen (Karlsruhe)
LB medium (Luria-Bertani broth)	+10 g/L BactoTrypton + 5 g/L Bacto Yeast Extract + 10 g/L NaCl pH adjusted to 7,5 with NaOH If needed 100 µg/mL Ampicillin is added to the broth	Invitrogen (Karlsruhe)
Carboxymethylcellulose Medium (CMC)	1,6 % CMC in L15 or DMEM (w/v)	-
cryoprotective medium	90% FCS 10% DMSO	-

A.5 Enzymes

Enzyme	Supplier
Benzonase	New England Biolabs® Inc. (Ipswich, USA)
DNAse Turbo DNA free	Invitrogen, Karlsruhe
HyQtase™ Cell Detachment Solution	Thermo Fisher Scientific Inc. (Waltham, MA, USA)
Platinum Taq-Polymerase	Invitrogen, Karlsruhe
Protease Inhibitor Cocktail Set VII Protease Inhibitor	Merck (Darmstadt)
Restriction enzymes SalI BamHI NotI XhoI	New England Biolabs® Inc. (Ipswich, USA)
RNasin® Ribonuclease-Inhibitor	Promega (Madison, USA)
RNAse Out (40 U/µL)	Invitrogen, Karlsruhe
SAP (shrimp alkaline phosphatase)	New England Biolabs® Inc. (Ipswich, USA)
Superscript® II Reverse Transkriptase (200U/µL)	Invitrogen, Karlsruhe
T4 DNA Ligase	Invitrogen, Karlsruhe
Trypsin	PAA Laboratories, Linz

A.6 Kits

Name	Supplier
BigDye® Terminator v3.1 Cycle Sequencing Kit	Applied Biosystems (USA)
IIFT Yellow fever virus	EUROIMMUN AG (Lübeck)
Invisorb® Spin Plasmid Mini Kit Two	Invitek (Berlin, Buch)
Invisorb® Plasmid Maxi Kit	Invitek (Berlin, Buch)
Invisorb® Spin DNA Extraction Kit	Invitek (Berlin, Buch)
NucleoSpin® Gel and PCR Clean-Up Kit	Macherey-Nagel (Düren)
NucleoSpin® Plasmid DNA purification Kit	Macherey-Nagel (Düren)
Pierce ECL Western Blotting Substrate	Pierce (Rockford, IL, USA)
QIAamp® Viral RNA Mini Kit	Qiagen (Hilden)
SuperScript® One-Step RT-PCR with Platinum® *Taq* DNA Polymerase	Invitrogen (Karlsruhe)

A.7 Primer, DNA and Protein marker

Primers used for the amplification of the gene of interest YFV-NS1

Name	Orientation	Sequence	Supplier
1960-1978	sense	GCGGATAACAATTCCCCGG	Invitrogen (Karlsruhe)
3311-3329	antisense	CCACCTTCTGATAGGCAGC	Invitrogen (Karlsruhe)

The primers were designed by Dr. Cristina Domingo Carrasco.

Primers used for the amplification of the gene of interest TBE-NS1

Name	Orientation	Sequence	Supplier
TBE E(TMD) - NS1	sense	GAGAGGATCCAGGGTTTCTACCAAAG	Invitrogen (Karlsruhe)
TBE E(nh+T2) - NS1	sense	GAGAGGATCCAGGCCTGAACATGAG	Invitrogen (Karlsruhe)
TBE E(T2) - NS1	sense	GAGAGGATCCAACAATGTCCATGAGC	Invitrogen (Karlsruhe)
TBE NS1	sense	GAGAGGATCCAGATGTTGGCTGCG	Invitrogen (Karlsruhe)
TBE NS1	antisense	GAGAGTCGACCGCAACCACCATTG	Invitrogen (Karlsruhe)

Primers used for sequencing

Name	Orientation	Sequence	Supplier
pTriEx-3 up	sense	GTTATTGTGCTGTCTCATCA	Merck (Darmstadt)
pTriEx-3 down	antisense	TCGATCTCAGTGGTATTTGTG	Merck (Darmstadt)

DNA marker

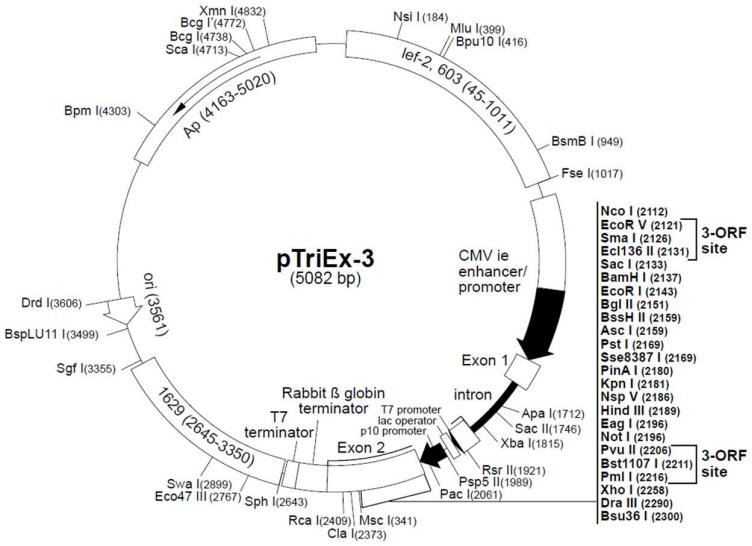
Name	Supplier
50 bp Ladder	Fermentas Generuler (St. Leon-Rot, Deutschland)
100 bp Ladder	Fermentas Generuler (St. Leon-Rot, Deutschland)
1 kb Ladder	Fermentas Generuler (St. Leon-Rot, Deutschland)

Protein marker

Name	Supplier
Prestained Protein Marker, Page Ruler	Fermentas Generuler (St. Leon-Rot, Deutschland)

A.8 Plasmids

Name	Supplier
pTriEx-3	Merck (Darmstadt)



A.9 Antibodies and Antigens

Primary Antibodies

Name	Target	Specification	Supplier
Anti-$(His)_6$-Tag	**$(His)_6$-Tag**	Rabbit	New England Biolabs® Inc. (Ipswich, USA)
mAB 6330 (monoclonal)	**YFV-E**	Mouse	[130]

Secondary labelled Antibodies

Name	Target	Specification	Supplier
Anti-Rabbit-IgG-HRP	**Rabbit IgG**	Goat	New England Biolabs® Inc. (Ipswich, USA)
YFV Anti-IgG-HRP	**Human IgG**	Rabbit	NovaTec (Dietzenbach)
YFV Anti-IgM-HRP	**Human IgM**	Rabbit	NovaTec (Dietzenbach)

Antigens used for ELISA

Name	Concentration	Supplier
E-III Domain	10 µg/mL	NovaTec (Dietzenbach)
E3 pp40	10µg/mL	NovaTec (Dietzenbach)
E3 pet44	10µg/mL	NovaTec (Dietzenbach)
Recombinant E-Protein	10µg/mL	NovaTec (Dietzenbach)
E-full 4T1	10µg/mL	NovaTec (Dietzenbach)
Recombinant NS1-Protein	1µg/mL	NovaTec (Dietzenbach)

A.10 Cell lines, viral and bacterial strain

Cell lines

Cell line	Temp./CO2	Description	Supplier
Vero E6	37°C/-	kidney epithelial cells derived from a monkey	ATCC # CRL-1586
PS	37°C/+	kidney epithelial cells derived from a pig	-

Virus strain

Name	Supplier
Yellow Fever Virus-17D	Vaccine strain Charge 354/1

Bacterial strains

Name	Genotype	Supplier
***Escherichia coli* One Shot® Top10**	F-mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 deoRaraD139 Δ(ara-leu)7697 galU galK rpsL (StrR) endA1nupG	Invitrogen (Karlsruhe)
RosettaBlue™(DE3) pLacI	F– ompT hsdSB (rB– mB–) gal dcm (DE3) pLacIRARE (CamR)	Merck (Darmstadt)

A.11 Software

Software	Supplier
BLAST	NCBI (Bethesda, USA)
EndNote8	Thomson ISI ResearchSoft (Berkeley, USA)
Lasergene® 8 SeqMan Pro Software (Version 8.1.5)	DNASTAR Inc. (Madison, USA)
MEGA 5	Center for Evolutionary Medicine and Informatics (Tempe, USA)
RTCA Software 1.2	Roche Applied Science (Mannheim, Deutschland)

A.12 Additional Results

In this chapter, some of the results obtained during the establishment of the YF 1gG and IgM ELISA's are shown. The first two figures (Fig. 61 and Fig. 62) depict results of a sample in different dilutions tested on the antigen supernatant of Asibi infected cells and NS1 producing cells also in different dilutions for the detection of YF IgG antibodies. The third figure (Fig. 63) depicts results of a sample in different dilutions on the antigen supernatant of NS1 producing cells also in different dilutions for the detection of YF IgM antibodies.

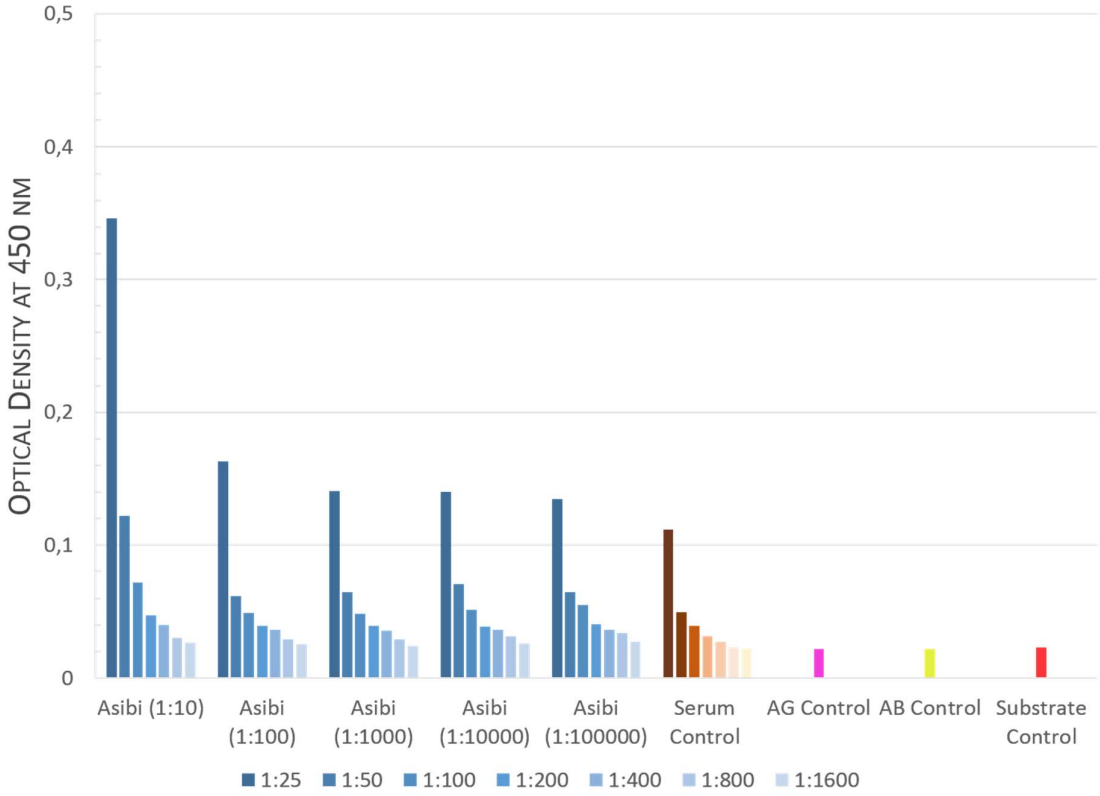


Fig. 61 OD values of ten-fold dilutions of the supernatant of Asibi infected cells starting from 1:10 to $1:10^4$. To these dilutions different serum dilutions are added (graded colour bars) in order to identify the best combination of antigen concentration and serum dilution. The bars in brown present the serum control (only serum no antigen). The colour gradation also marks the increasing serum dilution. The pink bar depicts the antigen control (AG control), the light yellow bar the antibody control (AB control) and the red bar the substrate control

As shown in the figure above (Fig. 61) absorbance readings of AG, AB and substrate control were all below 0.03 which was expected. The serum control had OD values ranging from 0.11 for the lowest serum dilution of 1:25 to 0.02 for the highest serum dilution of 1:1600. It is also noticeable that the antigen dilutions of $1:10^2$ to $1:10^4$ have all absorbance readings around the same value of the serum control, i.e. 0.1 to 0.15. Also, the intensity of the signal, i.e. the value for the absorbance reading declines with increasing serum dilution as is expected. As these signals have the same pattern and the same intensity as the serum control, all the signals of this antigen at dilutions $1:10^2$ to $1:10^4$ with the respective serum dilutions are considered as background signal. This is different for the antigen concentration of 1µg in combination with the lowest dilution of 1:25. Here the absorbance reading lies around 0.35. The rest of the serum dilutions show againb very low OD values ranging from 0.12 to 0.02 and hence can be considered as background signal.

The second antigen used was the supernatant of NS1 producing cells in different dilutions from 1:10 to $1:10^4$ per well. The results are depicted in the figure below (Fig. 62).

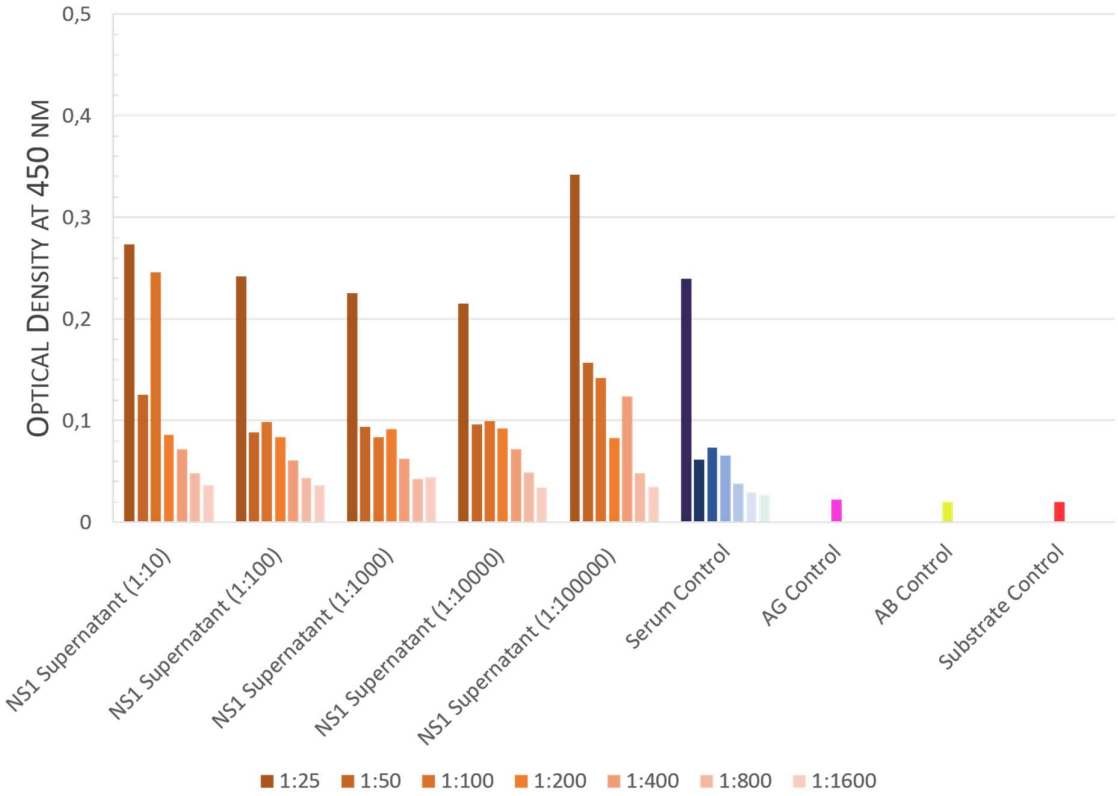


Fig. 62 OD values of ten-fold dilutions of the supernatant of NS1 producing cells starting from 1:10 to $1:10^4$. To these dilutions different serum dilutions are added (graded colour bars) in order to identify the best combination of antigen concentration and serum dilution. The bars in blue present the serum control (only serum no antigen). The colour gradation also marks the increasing serum dilution. The pink bar depicts the antigen control (AG control), the light yellow bar the antibody control (AB control) and the red bar the substrate control

As shown above, these bars do not look too different from the ones shown in Fig. 61. AG, AB and substrate control gave the expected results. However, the serum control in the lowest dilution of 1:25 showed a very high background signal of 0.24. The other serum dilutions do not give such a strong signal. However, the OD values of the other antigen concentrations with the respective serum dilution lie in the same range as the ones for the serum control. Hence, these signals can be considered as background signal. Furthermore, if the background signal of the serum control is substracted from the respective antigen dilutions, which have a slightly higher OD value than that of the serum control, the signals would still be too low (< 0.1) to be evaluated and considered for further use in the assay.

In the next figure (Fig. 63), results are shown, which were obtained during the establishment of the YF IgM ELISA. The same assay scheme as above was employed here as well – A positive sample was tested in different dilutions on the antigen of supernatant of NS1 infected cells (NS1 supernatant) also in different dilutions.

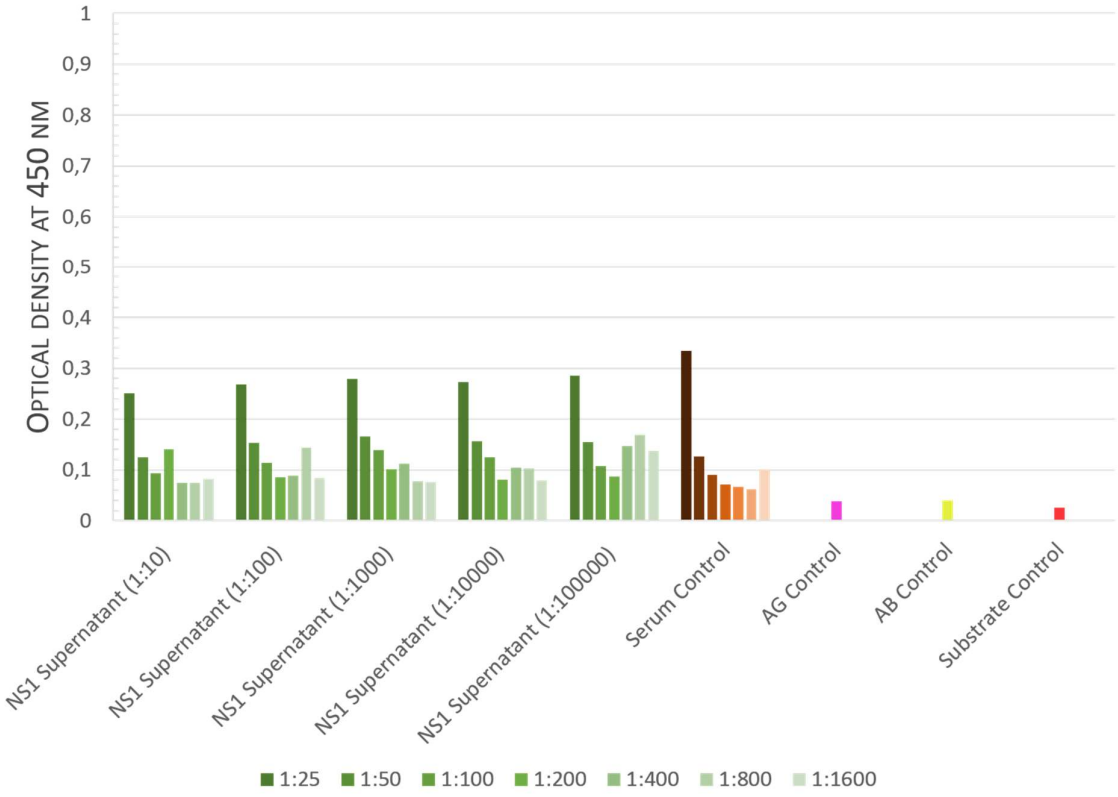


Fig. 63 Mean OD values of ten-fold dilutions of the supernatant of NS1 producing cells starting from 1:10 to 1:10^4 are presented here. To these dilutions different serum dilutions are added (graded colour bars) in order to identify the best combination of antigen concentration and serum dilution. The bars in brown present the serum control (only serum no antigen). The colour gradation also marks the increasing serum dilution. The pink bar depicts the antigen control (AG control), the light yellow bar the antibody control (AB control) and the red bar the substrate control

As can be seen absorbance readings of AG, AB and substrate control were all below 0.04. In general, the signal of the sample in all dilutions and antigen concentrations is very low (< 0.34). Furthermore, the gradation of the signals in the different antigen concentrations is not in accordance to the serum dilution in most cases. The serum control in the lowest dilution of 1:25 showed a very high background signal of 0.34. The other serum dilutions don't give such a strong signal. However, the OD values of the other antigen concentrations with the respective serum dilution lie in the same range as the ones for the serum control. Hence, these signals can be considered as background signal. Furthermore, if one would subtract the background signal

of the serum control from the antigen concentrations, which have a slightly higher OD value than that of the serum control, the signals would be still too low (> 0.1) to be evaluated and considered for further use in the assay.

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