Padlock Probe-Based Nucleic Acid Amplification Tests

Point-of-care Diagnostics of Infectious Diseases

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Abstract
Recent advancements in molecular biology and biotechnology have pushed the field of molecular diagnostics much further to benefit the society towards smart access for rapid and simplified health care. In this context, point-of-care (PoC) technologies that bring the inventions in diagnostics closer to bedside settings draw attention. This becomes all the more relevant in the case of infectious diseases which pose the major burden, in terms of mortality and economic loss, especially for third world developing countries with resource-limited settings (RLS). Moreover, emerging and re-emerging viruses, known for their rapid mutation rates, demand huge attention in terms of timely diagnosis and the need for effective treatments. Hence, appropriate and accurate tests to detect the pathogens with enhanced sensitivity and specificity would be needed to bridge the gap between bioanalytics and clinics.

This research work is an attempt to combine the tools and techniques required for the development of such efficient PoC technologies to combat infectious diseases. Among available nucleic acid-based amplification tests, padlock probing and isothermal rolling circle amplification are used to benefit from the advantages they offer for diagnostic applications, in terms of specificity, multiplexability, single molecule detection, high throughput, compatibility with various read-out platforms and inexpensive digital quantification.

In the first paper, simultaneous detection of RNA and DNA forms of adenovirus is shown to study the spatio-temporal expression patterns in both lytic and persistent infections. In situ quantification of viral DNA as well as transcripts with single cell resolution has been achieved. In the second paper, novel probe design strategy has been presented for the development of molecular assays to detect hypervariable RNA viruses. This approach becomes helpful in targeting rapidly evolving viruses by using mutation-tolerant probes for RCA. Third paper demonstrates simple RCA for rapid detection of Ebola virus in clinical samples, followed by a multiplexed detection with other re-emerging tropical viruses, namely Zika and Dengue. This study also includes a simple easy-to-operate pump-free membrane enrichment read-out, combined together with microscopy for digital quantification of the products. In the fourth paper, near point-of-care glucose sensor-based RCP detection has been proposed for Ebola virus detection. All these attempts clearly bring RCA closer to PoC settings for molecular diagnostics of virus infections.

Keywords: Nucleic Acid Amplification, Isothermal Amplification Methods, Padlock Probes, Rolling Circle Amplification, Molecular Diagnostics, Infectious Disease Diagnostics, Virus, Point-of-Care.
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Sibel Ciftci
To my beloved
Father, Mother & Sister

Canım Aileme
Knowledge without action is insanity, and action without knowledge is vanity.

Al-Ghazali
List of Publications

This thesis is based on the following papers:


*These authors contributed equally

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Related works by the author


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Introduction

Throughout the history of science, mankind has always contemplated the emergence of life as well as the complex structure and stunning function of living things. Efforts have been made to satisfy curiosities about life that already produced a remarkable amount of detailed knowledge and understanding of biomolecular events associated with health and disease. Deep thinking and scientific research, grounded especially in physics and chemistry, have unraveled how fundamental compounds can operate an interplay of biochemical reactions in a living being with striking processes. To decipher the mysterious complexity of life is, indeed, a daunting process and requires a holistic approach to understand it. In the past decade, tremendous progress has been made in order to cope with the intricacies of biological processes. Particularly, investigating life at the molecular level by analyzing DNA and RNA\textsuperscript{1,2} has spurred a growing interest towards genomic studies. Furthermore, the ramification of technological advancements empowered the research to a great extent and changed the face of biology, for instance from describing what we see by simply looking at images towards a more precise and predictive way. Likewise, molecular assays with integrated novel technologies have evolved into a potential opportunity to be used as diagnostic tools for biomedical practices aiding diagnosis and prognosis. Thus, recent advances in molecular diagnostics paved the way for a more personalized approach not only in clinical laboratories, but also in resource-limited settings (RLS). However, there are still major hurdles to tackle, and questions to be addressed before implementing such sophisticated technologies and biological assays in medical diagnostics. These include the kind of assays/technologies to be employed, the parameters of the tests to qualify them for biomedical/clinical applications such as accuracy, reliability, speed, sensitivity and specificity, together with practical considerations such as cost and user-friendliness. During my PhD work, I have addressed some of these challenging questions in order to develop molecular assays particularly for the diagnosis of infectious diseases.

The scope of this thesis (Figure 1) will focus on the exploitation of molecular assays and technologies used for nucleic acid detection, and their applications in infectious disease diagnostics. I will begin by giving a brief overview of existing molecular tools used for nucleic acid amplification, quantification and detection. The pros and cons of the available tools will be discussed throughout the thesis. Then, I will introduce the main focus of this work which
is the padlock probe-based rolling circle amplification assay. I will also address further the read-out platforms and microfluidic approaches for point-of-care (PoC) diagnostics. The second part of thesis will describe the assays developed during thesis work and will discuss how they addressed some of the aforementioned issues, together with a perspective note describing the future of molecular assays aimed towards PoC applications in RLS.

Figure 1: Overview of the scope of the current thesis, starting from sample collection and preparation, through isolation of nucleic acids and development of nucleic acid-based assays, to the integration of detection platforms towards point-of-care diagnostics.
Molecular Diagnostics: Need and Scope

Diagnostics play a crucial role in medical decision-making to deliver effective care by defining the source of patients’ problems. Early in the history of medicine, for instance, it was enough to examine a patient’s body in order to diagnose whether the patient had a fever or not. In course of time, clinicians obtained a better understanding of different diseases and their symptoms, and therefore could differentiate between different types of fever. In the 19th century, the invention of modern microscopy, and studies in histopathology and cytology increased our understanding of the biology of diseases at the cellular level. Furthermore, visualization of samples taken from a patient’s body using certain dyes and stains became possible and thereby provided not only identification, but also differentiation of cells and microorganisms. Thus, microscopic staining methods gave way to immediate prognosis and decision-making regarding treatment, however this has its own significant limitations.

Molecular diagnostics, in principle, begun with the term ‘molecular disease’ introduced in 1949 by Pauling’s finding of a single amino acid change in the β-globin chain causing sickle cell anemia. The following break-through in the discovery of DNA double helical model by Watson & Crick laid the foundation for molecular biology and also initiated the era of genomics. Subsequently, recombination DNA technology introduced during the 1970s paved the way for molecular diagnostics via nucleic acid hybridization methods (Southern blot) and sequencing. The former allowed the analysis of gene regions and was applied first as a prenatal diagnostic test for Thalassemia and other genetic diseases such as cystic fibrosis and phenylketonuria. However, constructing DNA libraries in order to identify disease-causing mutations was a cumbersome process and had significant technical limitations. Still, those advancements expanded our knowledge for understanding the root causes of disease and provided the first seeds for molecular diagnostics. The big transformation occurred with the invention of PCR that allowed molecular diagnostics to enter clinical laboratories as routine clinical tests. Indeed, soon after its invention, in 1987 Kwok and colleagues identified human immunodeficiency virus (HIV) for the first time using the PCR method in clinical diagnosis of infectious diseases. Molecular diagnostics entered its golden era during the 1990s when powerful technologies were developed for DNA sequencing and new genes were identified. In the last decade, continuous innovations led to massively parallel methods of whole genome sequencing and sequence databases within and between species that eventually led to the development of sensitive and specific diagnostic tools. Furthermore, advancements in nanotechnology and biotechnology allowed the integration of technology into current methods in order to meet practical challenges faced due to the complexity of the assays involved. As a result of this, the field has been shifted from being
manual towards greater automation, which also opened new vistas in personalized medicine and PoC diagnostics. Currently, molecular diagnostics are predominantly applied to genetic screening, detection of mutations, identifying inherited disorders, cancers, and infectious disease diagnostics. This thesis will mainly focus on approaches to rapid and sensitive diagnosis of infectious diseases in general, and virus diagnostics, in specific.

Infectious Diseases

Infectious diseases pose a rising global threat because of the quick spread of infectious agents across borders, causing a significant burden on economies and public health. Infectious diseases are caused by pathogens such as bacteria, fungi, mycobacteria, parasites, and viruses.

Outbreaks of smallpox, syphilis, cholera, tuberculosis (TB), and plague have resulted in millions of deaths throughout history. The earliest recorded devastating epidemic that occurred was the “plague of Athens (429-427 BC)”. The first pandemic, the “plague of Justinian (541-542)” was caused by Yersinia pestis and led to millions of deaths. Reaching its peak in the 14th century by the arrival of Bubonic plague known as “the black death”, Y. pestis returned, causing almost a third of the European population to perish. 3000 years old, smallpox had been one of the most deadly infectious diseases until the 20th century, and might have killed more people than all the wars in the history. Cholera outbreaks that occurred due to poor sanitation became a big concern in the 19th century. During the 20th century, humanity faced a shocking and terrifying disaster triggered by an influenza pandemic, also known as “Spanish flu”, that took 50 million people's lives.

Although infectious disease problems seem to be decreasing over the past decades owing to the development of vaccines, antibiotics, and methods aiding in better disease control and prevention, it still remains a major source of morbidity and mortality. Indeed, infectious and parasitic diseases are the second leading cause of disease as reported a quarter of annual deaths worldwide are attributed to infections. As seen from Figure 2, the global hazards of infectious diseases, that framed the basis for the Millennium Development Goals, need to be strategically addressed, especially in the poor third world countries facing enormous burden of these diseases. Over the past few decades, newly emerging and re-emerging infectious diseases have further deepened the global threats.
Recently, 87 species of pathogens have been discovered with an average rate of three to four new species emerging per year since 1980. Most of the current novel pathogens being recognized are viruses, in particular RNA viruses that originated from non-human reservoirs targeting a broad host range\textsuperscript{15}. The continuous global changes in ecosystem and contacts between humans and animals give infectious agents the opportunity to appear and overcome inter-species barriers to cause epidemics. Over time, pathogens evolve by undergoing genetic variation due to mutations, recombination, and assortment in order to adapt to their new ecological niches and hosts\textsuperscript{16}. Evolution plays a significant role in the emergence of new variants of existing pathogens and correspondingly novel infectious diseases.

Taking all these elements into consideration, a particular public health concern for the 21\textsuperscript{st} century is the alarming increase of illness due to emerging infections. Therefore, accurate identification of pathogens becomes a crucial consideration for efficient treatment and prevention of disease, in addition to developing effective therapeutics and preventive measures.

Figure 2. The burden of infectious diseases: The contribution of infectious diseases to global hazards, alongside other hazards shown in the world map; 84\% were outbreaks of infectious diseases; reproduced with permission from Ref. 14.
Emerging and Re-emerging Infectious Diseases

Emerging infectious diseases can occur either as instances of previously unknown pathogens, or as re-emergence of old foes that are continuously re-appearing in new geographic locations with rapidly increasing incidence and also in more pathogenic forms (Figure 3)\textsuperscript{17,18}.

The concept of emerging diseases became more prominent during the late 1960s with the sudden outbreaks of viral hemorrhagic fevers such as Ebola fever, Crimean-Congo hemorrhagic fever, and Lassa fever; however the most remarkable attention arose in the 1980s with the appearance of severe infectious diseases that caused huge epidemics such as HIV/AIDS\textsuperscript{10}.

Emerging zoonotic infectious diseases, those that emerge from wildlife and are subsequently transmitted to humans via contact with infected animals, are more prevalent in tropical countries. HIV infection and malaria arose from wild monkeys and originated in Africa\textsuperscript{19}, while mosquito-transmitted Zika infection recently re-emerged in Latin America, but was first documented in Rhesus macaque monkey in Uganda in 1947\textsuperscript{20}. Ebola virus, identified for the first time in 1976, has re-emerged to cause a string of incidents since 2001, causing its largest ever outbreak in 2013\textsuperscript{21}. Dengue, with a relatively recent evolutionary history, originally occurred in primates 1000 years ago, and has re-emerged periodically during last few hundred years. It currently threatens almost one third of the global population\textsuperscript{22}. Their transmissions first occurred

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{world_map.png}
\caption{World map showing the increased accumulation of newly emerging (red) and reemerging (blue) infectious diseases since early 1980s and also before, deliberately emerging (black); reproduced with permission from Ref. 17.}
\end{figure}
from animal reservoirs to humans, and further spread from humans to humans\textsuperscript{23}. Over time, infectious pathogens pass through various stages in order to adapt to their changing environments prior to causing epidemics. Emerging infections have always been a burden to humanity throughout history and are continuously posing global health risks with serious social, political and environmental effects. Therefore, emerging infectious diseases deserve special attention and fast action at all times. The first step to rapid response is rapid identification.

\textit{The role of RNA virus evolution on emerging infections}

Viral pathogens are the most remarkable root cause to emerging infections, having caused two-thirds of infectious diseases leading to significant outbreaks such as Ebola, Flaviviruses (Zika, Dengue, West Nile, etc.), and SARS\textsuperscript{10}. Among those viral pathogens, RNA viruses make up the greatest proportion as there are nearly four times more RNA virus species than DNA virus species in existence, and these take the greatest portion among other recently discovered pathogens\textsuperscript{24,25}.

RNA viruses can be classified according to their mode of mRNA production as follows: double-stranded RNA viruses like rotavirus (gastroenteritis); single-stranded positive sense RNA viruses like picornaviruses (common cold, hepatitis, meningitis), and flavivirus (zika, dengue); single-stranded negative strand RNA viruses like rhabdovirus (rabies), filoviruses (Ebola) and orthomyxoviruses (human influenza virus); single-stranded RNA viruses with reverse transcriptase (mRNA is produced from the integrated DNA that is reverse transcribed from the viral RNA genome) such as retroviruses (Human Immunodeficiency Virus (HIV))\textsuperscript{26,27}.

The highly variable viral RNA genome, due to high error rates, is believed to benefit virus populations by helping them to quickly adapt to their changing environmental and biological niches. RNA viruses exhibit higher evolution rates and more heterogeneous populations than DNA viruses mainly due to high mutation frequencies of the RNA genome. The shorter replication time of RNA viruses and lower fidelity of RNA viral polymerases create the main differences between the evolution rate of DNA and RNA viruses. Several different methods strongly suggest that the viral RNA genome has a million-fold higher mutation rate than DNA viral genome\textsuperscript{28}. It is also well known that RNA polymerases are more error-prone as they lack effective proofreading mechanisms, which is not the case for DNA polymerases. Indeed, DNA polymerases produce between $10^{-7}$ and $10^{-11}$ error rates per base per replication while RNA
polymerases produce $10^{-4}$ errors per site during a limited number of RNA replications\textsuperscript{29}. The decreased fidelity of RNA polymerases allows RNA viruses to have higher fitness and pathogenicity under selective pressures\textsuperscript{30}.

In addition to the rapid evolution rate due to low polymerase fidelity, RNA virus genomes are also exposed to reassortment and recombination processes which may allow for large evolutionary jumps. In case of RNA viruses with segmented viral genomes that contain one or more viral genes, they have the ability to produce new viruses in a host infected by a mixture of different strains of virus. Through this process, highly virulent viruses can be produced out of low-virulence, segmented RNA viruses such as lymphocytic choriomeningitis virus (LCMV) and arenavirus, which due to their combined reassortment showed high virulence, causing lethal disease in mice, while parental strains and reciprocal reassortment did not\textsuperscript{31}. Thus, the reassortment process of segmented viral RNA genes plays a significant role in the emergence of new infections by generating highly virulent and antigenically novel pathogens. Gene rearrangements other than point mutations such as duplications, deletions and insertions intensively take place during recombination with other viral or cellular genes. Although the process is used by a limited number of standard RNA viruses, it is quite common among retroviruses. This event not only generates new virus diversity but also plays a significant role in RNA virus evolution\textsuperscript{29}.

The heterogeneity of RNA virus populations is quite well known. Sequencing studies have reiterated the great heterogeneity of viral populations\textsuperscript{32,33}. Sequences of virus isolates obtained at different times from a single patient were not alike, suggesting that they had diverged from the original ancestor\textsuperscript{34}. In other words, an infection caused by a wild-type virus can generate a mixture of new antigenic variants. The most favorable variants out of the predominating ones will be selected once the environmental conditions change and will go on to generate viable quasispecies\textsuperscript{35}. It is noteworthy to mention that sometimes very few, or even a single mutation, can result in significant changes in virulence phenotype or in a broad range of host organisms. Such an event happened in the 1983 chicken outbreak of an influenza A type virus that became virulent with only a single point mutation of an avirulent form, causing 80% mortality in the infected population\textsuperscript{36}. Hence, viruses, particularly with RNA genomes, due to their extremely high mutation rates, have the ability to rapidly evolve, coupled with enormous genetic variability, thus creating a great potential for initiating unpredictable disease outbreaks.

RNA viruses engender most of the newly emerging infectious diseases due to their inherent high error rate and rapid evolutionary capacity when compared to DNA viruses or other pathogens\textsuperscript{37}. The extremely heterogeneous viral RNA populations, which are composed of quasispecies variants, may undergo
rapid evolution under certain conditions and may result in severe diseases that are previously unknown, as in the case of HIV/AIDS\textsuperscript{38}. Additionally, these sophisticated evolution processes used by RNA viruses in order to adapt and survive create a great challenge to develop effective vaccines and diagnostic tools.

Molecular Diagnostics for Infectious Diseases

Molecular diagnostic tests today are widely used in research labs, reference labs, clinical, and PoC settings for precisely identifying infectious diseases. Infectious diseases are the fastest growing and the most dominant area in the molecular diagnostics field. Indeed, infectious disease applications constitute the highest share (50-60\%) in the global molecular diagnostic market, greater than other areas such as oncology, genetic testing, and even blood screening or glucose testing\textsuperscript{39,40}. A good molecular diagnostic test for an infectious disease should be able to detect the pathogen whether it is a virus or bacteria, identify its different strains, and identify whether it has antibacterial resistance, or is avirulent. It should also quantify the concentration of the pathogen to inform whether or not it is responding to treatment.

However, available diagnostic methods are still lacking in terms of specificity, sensitivity, long turn-around times from sample to result, inaccessibly sophisticated tools, and limited throughput capacity. Furthermore, the complex nature of pathogens contributes more to those limitations. Undoubtedly, clinicians face many hurdles to assess infectious diseases due to lack of available “ideal” diagnostic tools. This is particularly true for acute infections or outbreaks where clinicians are obliged to diagnose the disease immediately to prevent further spread of the pathogen and also treat the patient properly. The first assessment a clinician usually performs is the observation of symptoms via a physical examination and verification of medical history in a primary care setting. Exceptional cases may require escalation to expensive and time-consuming laboratory investigations. In addition, various pathogens may share common symptoms, for example influenza and obscure diseases, or be completely asymptomatic only to be detected serendipitously during health screens or during unrelated consultations. As such, diagnosis may not be pathogen specific, and clinicians may end up prescribing improper medications such as broad-spectrum antibiotics. The consequence of this is today’s serious global problem with “anti-microbial resistance”. Diagnosis is also troublesome for infectious diseases that are highly contagious and have the potential to cause outbreaks such as Ebola Virus Disease (EVD). Initial symptoms of Ebola infection are not specific, usually exhibiting flu-like symptoms\textsuperscript{41}. Therefore, its symptoms can be easily confused with other commonly occurring infections in those regions such as malaria. It goes without saying that an
Ebola infected patient should be isolated from healthy individuals immediately in order to prevent spread that can quickly escalate to epidemic proportions. It is also possible that malarial infection and Ebola infection can occur simultaneously while waiting for a diagnosis. Pathological changes may appear much later than the disease process has been triggered. This latency period may differ from disease to disease, for example, SARS within 6 days, Hepatitis B 1-6 months, HIV from a year to 15 or more, during which patient carries the virus without being aware of it, while still being able to transmit it\textsuperscript{42,43,44}.

In the following sections, I will give an overview of how infectious diseases are being diagnosed in different lab settings, and also a glimpse into the future of molecular diagnostics in the infectious diseases field.

\textit{Molecular Biomarkers}

Biomarkers are biological molecules that can be measured and used as unique indicators to evaluate normal biological processes, pathogenic conditions, and responses to treatments\textsuperscript{45}. These molecular signatures play a vital role in disease diagnostics, therapeutic tests, and now it is also growing towards the field of personalized medicine. A good biomarker is capable of relating a unique biological trait attributed to a specific disease condition. Moreover, biomarkers can reveal information from the very early stage until the terminal stage of a disease. Many biological processes taking place in our bodies during normal or abnormal states, as well as healthy or unhealthy conditions, are mainly associated with genes and proteins, and their interactions with each other. Therefore, being able to evaluate an individual's health condition at the molecular level, detecting and measuring specific genetic sequences in DNA and RNA or the proteins they express, becomes crucial.

Many methods and techniques are built upon one of these biomarkers for disease diagnostics. However, selecting the right biomarker for a detection method is the most fundamental step in the process, and also crucial for developing molecular technologies. An optimal biomarker method requires accurate and precise detection, with sufficient specificity to differentiate between multiple molecules of closely related species, as well as high sensitivity to detect low-abundance molecules.

Proteins are the most common type of biomarkers used in the diagnostics field. In infectious disease diagnostics, for instance, the most prominently used protein biomarkers are antigens produced by pathogens, and antibodies produced by the patient as an immune response during infection. Nearly all protein detection methods rely on antibodies used for virus detection. However, quality and availability of antibodies can be a limiting factor. Moreover,
antibodies very often show cross-reactivity with similar proteins of different species, or even exhibit unpredictable cross-reactions. The sensitivity of an antibody-based detection method can also be challenged due to low expressing proteins. Not just antibody, but also protein biomarker availability can be limited since protein coding genes account for only a small proportion of our genome\textsuperscript{46}. It is also well known that proteins do not always show linear correlation with RNA levels, which might be crucial in evaluating the state of a biological system, but may not be reflected on the protein level\textsuperscript{37}. Thus, measuring such proteins becomes irrelevant, as they are, in fact, not necessarily directly associated with the biological state. On the other hand, there may be physiological conditions or infections in which nucleic acid biomarkers remain unchanged, but their final functional product, proteins, may alter. Although there are well-established methods available, protein biomarkers still suffer from variabilities in detection that will be addressed in the following diagnostic methods section.

Recent advancements in sequencing technologies (such as NGS) have provided novel nucleic acid biomarkers that are of benefit in tackling challenges and limitations encountered by protein biomarkers. Nucleic-acid based methods in particular use nucleic acids as biomarkers; both DNA and RNA. Since DNA and RNA are the carriers and transmitters of genetic information, gene alterations such as single nucleotide variation (SNV), copy number variations (CNV), structural anomalies, and gene regulation can reveal more insight for biological conditions and for early diagnostics. The ability of nucleic acids to be amplified allows for high sensitivity detection even in trace amounts compared with protein biomarkers. In the scenario of infection, some viruses causing long-term infection, for instance HIV, may not produce viable or infectious viral particles but it keeps a silent reservoir of its genome in host cells. In that case, detecting protein biomarkers will most likely hinder a proper diagnosis; however, viral nucleic acids detection will alert to the existence of the pathogen and also provide an early possibility for diagnosis.

**Conventional vs. Molecular Methods for Viral Diagnostics: From Culture to Genome**

To this date, clinical laboratory settings have many available methods for pathogen detection with varying sensitivity and specificity. However, the first PoC health contacts, such as primary health centers, that serve most populations, are lacking such lab-based resources.

Diagnostic methods that are established and commonly utilized in lab-settings for infectious diseases can be categorized as conventional and molecular.
Overall, these methods can be further partitioned into six general types: (1) confirming the presence of a pathogen; (2) direct visualization of pathogens; (3) detecting pathogen via antigen-antibody response; (4) detection of antigens; (5) direct detection of nucleic acids of pathogens; (6) PoC rapid diagnostic methods. Diagnostic methods, in general, should be able to produce reliable and useful test results that can be achieved by using accurate, robust, sensitive, and specific methods. In addition to those crucial prerequisites, speed, simplicity and low-cost become desirable especially for labs in RLS.

The current state of clinical labs for the infectious disease diagnostics is in transition and they accommodate both conventional and novel technologies. In the following sections, my thesis will give an overview about conventional and modern molecular methods available for infectious disease diagnostics with a main focus on virus detection and the most recent advancements towards their implementation in PoC settings.

**Conventional molecular methods for pathogen detection**

Today, there are plenty of conventional methods available that are widely used in well-equipped clinical microbiology labs which can speed up the suitable treatment for patients, preventing the spread of infection, and monitoring drug responses. Even though traditional methods suffer from certain limitations, such as being cumbersome and slow, they are, on the other hand, well-established and cheap. Moreover, traditional methods still remain as “gold-standards” to which new methods need to be compared.

For a long time, clinical labs have relied on culture and microscopy based-methods as the first line of diagnosis for infectious diseases\(^4^8\). The combination of these techniques remains the mainstay of rapid pathogen identification and also complements other conventional methods. Culture methods require growing pathogens in appropriate media such as agar and cells. In particular, shell vial culture is one of the most widely used methods for virus isolation and identification where virus particles are centrifuged onto a single layer of cells and viral growth is subsequently measured via antigen detection. An unknown virus from clinical samples can be grown in cell cultures and the identification of this virus can be made by observing morphological changes in cells as a result of its cytopathogenic effect. Moreover, a single viable viral particle can be further expanded in cell cultures to yield sufficient material for further examination by other diagnostic methods, for instance immunofluorescence, immunohistochemical staining, antigen-capture Enzyme Linked Immunosorbent Assay (ELISA), microscopy, and nucleic acid amplification tests such as Polymerase Chain Reaction (PCR), Rolling Circle Amplification (RCA), Loop-mediated Isothermal Amplification (LAMP), etc. However,
only live viruses can be grown in cell cultures. Therefore, patient sample deterioration during transportation may have profound effects on virus viability and consequently on the test results. On the other hand, culturing is only applicable to those viruses that can be grown in labs; it is not amenable for highly pathogenic viruses such as Ebola that require high safety biocontainment laboratories. Furthermore, not all viruses can grow in cultured cell matrices, such as norovirus and hepatitis virus. Most importantly, the time required for preparing and maintaining cultures, as well as the growth phase of pathogens, are the main challenges faced by culture-based methods.

Another classical method extensively used in well-developed clinical labs is advanced microscopy in which pathogens can be directly visualized and also morphologically identified. Light field microscopy of wet mounts is easily accessible in RLS, but more informative and sensitive microscopy methods may be out of their reach. Electron microscopy, for instance, can provide an immediate diagnosis even for non-cultivable or non-viable pathogens. It also allows a rapid identification of novel pathogens. However, electron microscopy is expensive, and therefore not available in many settings, and requires sophisticated equipment that not only requires highly skilled personnel but more importantly suffers from low detection limits (10⁶ virion/mL fluid matrix). Eventually, those two gold-standard methods have begun to be replaced by more sensitive, specific, and less-time consuming molecular methods that are becoming the frontrunners of pathogen detection in clinical labs. In recent years, ‘immunological methods’ have been widely used for the diagnosis of many infectious diseases. Those methods rely on antibody-antigen interactions in two main ways: direct detection of antigen represented by a pathogen, and detection of immune response to a pathogen. The latter, as seen in ‘serological methods’, detect the host antibody response triggered by foreign molecules, namely antigens. Serology tests may also assist in determining patients’ exposure status to a pathogen depending on IgM and IgG antibody levels; early and late stages of infection, respectively. Enzyme immunoassay (EIA) or ELISA is one such serology method that allows detection of pathogen-specific antibodies in serum on a solid matrix coated with antigens of the pathogen. Principally, antibodies present in serum will bind to their antigens and can be detected visually using enzyme tagged with species-specific antibodies that can produce colour upon enzyme-substrate reaction. By the use of analytic detectors i.e., spectrophotometer and monoclonal antibodies, specificity and sensitivity in antibody detection can be further enhanced. IgM ELISA, in particular, is a pillar of serologic diagnosis of acute infections by capturing IgM antibody highly present at the sensitization stage. Virus specific antibodies can also be detected and quantified by a serum (virus) neutralization assay. In principle, virus-specific antibody containing serum neutralizes the virus, hence inhibiting the cytopathic effect of the virus.
amount of the antibody in the serum can be determined through serial dilutions; the dilution that initiates neutralization is considered as the titre of the serum. Neutralization assay is slow, requires virus production, and is technically demanding. On the other hand, it is species-independent and very feasible for isolating new agents within a few weeks, unlike EIA tests which are species dependent and their development and validation may take months to years\textsuperscript{50}. For identification of immunogenic proteins and monitoring their full antibody profile, \textit{immunoblotting assays}, such as western blot, are preferable. Even though there are routine tests in clinical settings, immunoblotting assays are often used as confirmatory tests for ELISA results. More rapid detection and quantification of antibodies can be achieved by \textit{indirect immunofluorescence assay (IF)}. This assay requires virus-infected cell cultures fixed on a solid phase to test serum containing antigen-specific antibodies. Their binding can be visualized using fluorescently labelled anti-species secondary antibodies. IF assays provide test results within two or less hours with specific and sensitive viral identification\textsuperscript{50}. However, it may fail to confirm all viral strains due to lack of sensitivity and possible cross-reactivity of antibodies. Certain viruses such as paramyxovirus, coronavirus, adenovirus, and influenza viruses have the property of precipitating red blood cells via binding of hemagglutinin protein present on viral capsids with red blood cell receptors. To mitigate this effect, the assay includes a feature called a \textit{haemagglutination–inhibition assay}\textsuperscript{50,52}. The existence of antigen-specific antibodies in serum prevents the agglutination process and forms a compact button of RBCs. This method still remains as the gold standard for detecting antibody responses to avian and influenza A viruses\textsuperscript{53}. In principle, the assay is simple but laborious, and cannot differentiate the phase of infection since agglutination occurs as a result of either IgM or IgG antibodies. The last, but most traditional serological method of virology diagnostics, is the \textit{complement fixation test (CFT)}. Complement, a serum component, reacts only with antigen-antibody complexes. Thus, the complex prevents the complement from interacting with RBCs coated with anti-RBC antibodies, used as an indicator, and remains intact giving a positive test result. CFT is used as a reference method for validating new serological tests. However, it is too complicated and demanding of a procedure to be used in clinical diagnostics.

Identifying infectious agents and the cause of disease by testing immune response is problematic. Because every individual's immune system is unique, the antibody composition of each patient is subjected not only to genetic differences, but also to the influence of the various infectious pathogens encountered previously. Therefore, serological test results can show significant variation and are difficult to standardize in different lab settings. Moreover, antibody testing often results in false-negative results due to cross-reactivity with other pathogens, vaccination and autoimmune diseases\textsuperscript{54,55}. Serology testing
of infected immune-compromised patients often fails due to inadequate antibody response. Serological diagnostic tests are usually not significant until there is an immune response and that might take several weeks or even months, depending on the pathogen and the previous exposure profile of the patient, even after clinical symptoms appear. Furthermore, serology cannot distinguish active, past, or asymptomatic infections. Overall, serology is not preferable as a stand-alone test for diagnosis and decision making for treatment, but rather can be used along with other diagnostic methods for confirmatory testing.

As an alternative to antibody testing, other immunoassays for ‘direct detection of viral antigens’ have also been in use in clinical labs today. These methods challenge serology tests due to their enhanced sensitivity and specificity and reduced assay turn-around time. Unlike serology tests, antigen-testing immunoassays are applicable to a wide range of specimen types such as tissues, cells, secretions, blood and excretions. Viable virus and the intact form of the virion are not necessarily required for direct antigen detection, it works better with non-structural proteins which are present abundant in infected specimens. Antigen in a tissue sample or cultured cells can be directly detected with immunofluorescence staining or fluorescent antibody staining. This becomes possible using antigen-specific antibodies conjugated with fluorochromes that can be visualized with IF microscopy. Based on this technique, pathogens can be detected in their natural environment (in situ) and thus specific localization of antigens assures a legitimate diagnosis. Similarly, immunohistochemical staining allows antigen detection using enzyme tagged antibodies that can produce colors upon reacting with their substrate and visualized using light field microscopy. Even though this technique is slower than IF staining, it is of great benefit in better understanding of lesions or deformations in tissue structure that are attributed to specific viruses. Direct and indirect ELISAs (sandwich and competitive modes), however, revolutionized infection diagnostics, and have become the most reliable and widely used methods mainly due to their simplicity and short waiting time for conclusive results compared to the classical serology tests. The most typical format is a solid-phase EIA where antiviral antibodies are immobilized on a solid surface that allows for the capture of virus or viral antigens present in infected specimens. This way, only captured antigens can be visualized via labelled antibodies that can produce a discernible signal to be evaluated with the naked eye (colour change), or via various detection methods such as spectrophotometry, fluorescence, or chemiluminescent measurement. Sandwich ELISA provides higher sensitivity and specificity than other conventional immunoassays. It does not require antigen purification and is therefore more feasible to analyze complex specimens. The limiting factor can be standardizing the assay and optimization of selected antibodies to reduce cross-reactivity in the signals.
**Viral Nucleic Acid Detection**

Early diagnosis of viral diseases is crucial in order to receive proper treatment and also to prevent potential transmission, which can escalate to larger outbreaks. Methods with high sensitivity, specificity, and accuracy play an important role in early diagnosis. In order to understand the importance of diagnostics, particularly in the early phase of infection, let us consider HIV infection as an example. Acute or primary phase infection is when the virus has just established itself in the host and is starting to propagate becoming highly infectious. During this phase, the immune system has not yet come into play, but the number of HIV RNA copies reach their peak and the new viral particles are released into bloodstream\(^{60}\). Within a few weeks or months, body defense mechanisms spring into action and start producing antibodies due to the high viral load, known as seroconversion phase. Eventually, infection enters the long-term phase during which virus replication continues asymptotically, lasting up to ten years and if it remains untreated, symptomatic AIDS develops resulting in life threatening HIV-related opportunistic infections\(^{61}\). Patients may live for many years without being aware that they have had the virus, which poses a great risk for transmitting the virus, as well as delaying a proper treatment.

![Figure 4: Schematic representation of the various phases of a viral infection. Serology testing becomes possible only during the late phases where there is a prominent antibody response. Likewise, viral antigen detection becomes possible only after the onset of the symptoms. Henceforth, pathogen detection](image-url)
**during the early stages of the infection, by virus isolation or NAT, which marks the early diagnostics is the need-of-the-hour.**

Diagnosis at early/acute phase becomes critical (Figure 4) as the patient is very infectious and most likely unaware of being infected due to lack of symptoms or have very few flu-like symptoms which only start to appear when seroconversion occurs. However, most of the standard tests are HIV antibody tests that can only be used after one to three months of infection\textsuperscript{62,63}. Moreover, the tests at the early phase of infection are likely to produce false-negative results despite the presence of infection\textsuperscript{64}. More recent traditional tests based on virus antigen detection (ELISA) can detect infection earlier, even within two weeks of infection\textsuperscript{65}. Nevertheless, there are certain conditions such as Lyme disease, syphilis and lupus that can show positive results for ELISA HIV tests\textsuperscript{66,67}. Thus, another follow-up test, usually Western blot, is used for further confirmation. Besides, HIV exists as two main types, HIV-1 and 2, both of which have multiple groups which branch out as subtypes, and further as strains\textsuperscript{68}. This genomic diversity in the virus acquires different properties affecting the speed of transmission, response to drugs, and prevalence in different geographies. Therefore, not only detecting the presence of HIV is important, it is also imperative to be able to identify which subtype and strain is causing the disease for prevention and treatment. However, most traditional methods depend on identical morphologic or metabolic properties rather than genetic diversity that exists among different strains of the same virus. Taking these into consideration, detection (presence of virus), identification (subtypes/strains) and quantification (viral load), high specificity (only the target of interest), sensitivity (measurable lowest quantity) and precision (reliable reproducibility) at the earliest stage of infection have become the most desirable parameters for diagnostics.

To alleviate the limitations affecting conventional immuno-based methods, nucleic acid-based tests (NATs) have become an increasing trend in the diagnostic field over the past decade. Nucleic acid detection methods provide specific solutions to several drawbacks in the aforementioned methods such as, (1) difficulty in culturing viruses (2) non-viable or deformed viruses subjected to mishandling (3) difficulty in clinical identification of asymptomatic viruses or persistent infections in which viral DNA/RNA exist in undetectable but potentially infectious form (4) challenges in investigating nucleotide variations that are associated with drug resistance (5) when identifying closely related viruses that have identical morphological features is desired (6) inability to detect emerging new viruses that have not been known previously. Nucleic acid amplification tests (NAATs) use genetic materials (DNA, RNA) as biomarker targets which can be detected with hybridization, amplification and sequencing technologies.
Hybridization techniques have been used in many assay formats using extracted nucleic acids and *in situ* pathogen detection. One such method, fluorescence *in situ* hybridization (FISH), allows direct nucleic acid detection of pathogens in their natural host environment (*in situ*) using fluorophore labeled probes. Upon hybridization of labeled probes, genes of interest can be visualized under fluorescent microscopy. The FISH assay has already been described for the detection of various pathogens, such as identification of antibiotic resistant *Helicobacter pylori* from biopsy samples, *Plasmodium sp.* in blood smears, replicating genomic Dengue virus RNA in insect salivary gland tissue, and persistent asymptomatic Ebola virus infection\[^{69,70,71,72}\]. As a microscopic and *in situ* technique, FISH assays provide spatial information and pathogen identification in co-infected samples. Considering its modest technical requirements together with low-cost and rapid identification of pathogens (approx. 45 min.) with good specificity, FISH has already been used in RLS\[^{70}\]. However, it suffers from poor sensitivity and becomes particularly challenging when attempting to detect low abundant targets. FISH has thus become outdated and been replaced with more advance technologies such as MALDI-TOF-MS and *next generation sequencing* in many developed clinical settings. However, it still remains as a useful technique in adequately equipped lab settings where other more sophisticated methodologies remain inaccessible or impractical.

**Nucleic Acid Amplification Testing (NAAT)**

Nucleic acid amplification testing (NAAT) has become an outstanding approach to shorten the extended time window for diagnosis of infected patients via immuno-based tests. NAAT generates immense amounts of nucleic acid by amplifying minute amounts of sample. As a result, the sensitivity increases profoundly, which makes it possible to detect pathogens soon after infection or even before the onset of clinical illness\[^{73}\].

*Polymerase chain reaction (PCR)* is one such conventional nucleic acid amplification technique that can exponentially increase the number of copies of selected target genes through a repeated number of cycles of rapidly altering temperatures. Thermal cycling exposes reactants to repeated cycles of heating and cooling to permit different temperature-dependent reactions, specifically DNA melting and enzyme driven DNA replication. An essential set-up for PCR includes: a PCR machine, which can provide temperature ramping, a target to be amplified, short oligonucleotides or primers complementary to the target sequence, a thermostable DNA polymerase and a suitable chemical environment for optimum enzyme activity and stability. In principle, PCR involves three major events, namely template denaturation, primer annealing
and extension of annealed primers by DNA polymerase. A number of repeated cycles of these steps yield an immense accumulation of the target, approximately a several million-fold amplification in an hour. While exponential amplification increases the sensitivity in a short time, the specificity of PCR is achieved by the sequences of primers that are complementary to the selected target. Amplification only occurs between the two bound primers, so both primer sequences have to match and successfully anneal to their targets. Besides amplification, primers can also be used as barcode sequences for detection and identification of the amplified targets in a mixture of complex reaction. Moreover, the diagnostic sensitivity can be increased by the inclusion of mismatched bases in defined positions that can permit detection of genetic variants of a pathogen. This break-through advancement of PCR in clinical diagnostics began with the invention of real-time PCR, a quantitative assay for amplified PCR (qPCR) products being generated in real time. This became possible using fluorescent intercalating dyes or fluorescent labeled probes that continuously hybridize to products during amplification cycles, thus allowing quantification of initial products rather than end products generated with conventional PCR methods. One important variant of qPCR is reverse transcription polymerase chain reaction (RT-PCR) that can quantitatively detect (RT-qPCR) RNA through complementary DNA (cDNA) generated by reverse transcription. This method enables effective detection and quantification of RNA viruses. Further developments enhanced the multiplexing capacity of PCR; hence simultaneous amplification and detection of multiple targets using several primers and different fluorescent dyes in the same reaction mixture became possible. A combination of real time and multiplex PCR has become useful in the evaluation of complex infectious diseases caused by for instance co-infections and/or different subtypes of virus.

The various forms of PCR have been accepted as widely used approaches in clinical settings and even became gold-standard techniques for diagnosing and monitoring certain infectious diseases or as confirmatory methods for novel techniques. The main advantages that PCR offers are as follows: 1) it eliminates culturing pathogens thereby allowing detection of non-cultivable or slow-growing pathogens as well as non-viable and highly infectious pathogens; 2) rapid and high sensitivity in comparison to conventional immuno-based methods; 3) simultaneous detection of several pathogens; 4) enabling quantification needed for monitoring viral load. However, the major problem often encountered with PCR methods is high risk of false negative or false positive results caused by contamination, mismatched primers and target sequence, altered experimental conditions, inhibitors carried over by the isolation of nucleic acids, and incompatibility with suitable detection platforms. Even though, it surpasses conventional methods in many ways, PCR as a method still faces many hurdles that requires a well-designed assay and careful interpretation of results. PCR platforms can be easily established in well-
developed clinical settings, while they may not be feasible in RLS for PoC use due to their limited power supply and lack of contamination-free spaces. Figure 5 provides an overview of the comparative features of both conventional techniques and NAAT-based methods used in different types of settings such as primary health centres, hospitals, reference labs, PoC, etc., for viral diagnostics.

Figure 5: Comparative picturesque overview of conventional and nucleic acid-based tests used in viral diagnostics, with the instruments or techniques used, duration range and utility in diverse settings.

Alternative methods to PCR for resource-limited settings: Isothermal Nucleic Acid Amplification

Pathogens causing infectious diseases are a particular burden in developed or underdeveloped nations that cannot afford expensive technologies, and
have restricted resources that do not allow for adopting sophisticated instruments such as thermal cyclers needed for PCR. Isothermal amplification of nucleic acids has emerged as an alternative method to alleviate the challenges faced by PCR in RLS. Isothermal amplification allows a rapid and efficient amplification of nucleic acids at a constant temperature without the need for complex instrumentation. On the contrary, constant temperature needed for isothermal amplification can be easily supplied using simple heat generators such as water baths, resistive heaters or by exothermic chemical heating. In the past two decades, numerous isothermal amplification methods have been developed, most of which achieved very efficient and sensitive nucleic acid detection. Taking advantage of the simplicity and efficiency of isothermal amplification methods, attention has now shifted towards developing simple and rapid detection assays for PoC, including bedside diagnostics. With the recent advancements of microfabrication, developments in diagnostics began to strive for integrating isothermal amplification methods into various assays and read-out platforms such as microfluidic chips, paper-based tests and biosensor technologies.

The existing isothermal nucleic acid amplification methods can be divided into three categories based on their reaction kinetics: (1) exponential amplification such as nucleic acid sequence-based amplification (NASBA), strand displacement amplification (SDA), loop-mediated isothermal amplification (LAMP), helicase-dependent amplification (HAD), recombinase polymerase amplification (RPA) (2) linear amplification such as rolling circle amplification (RCA) (3) cascade amplification such as circle-to-circle amplification (C2CA). All these isothermal amplification methods rely on distinct enzymatic activities, and primers or probes, which do not require heating and cooling cycles for the amplification to occur. There are strengths and weaknesses for each of these methods and they differ in terms of sample preparation, mode of amplification and detection, and features like sensitivity, specificity, and assay run time. Therefore, there is no one best method that suits all conditions, but rather provides alternative options according to situational needs.

**Nucleic Acid Sequence-Based Amplification (NASBA):**

NASBA, also known as “Self-Sustaining System” (3S), is a transcription mediated isothermal nucleic acid amplification method. NASBA amplifies single stranded RNA templates using reverse transcriptase and T7 RNA polymerase. In principle, targeted single stranded RNA is converted to double stranded cDNA using primers with a T7 promoter and reverse transcriptase. This cDNA acts as a template for T7 DNA dependent RNA polymerase to make multiple copies of antisense RNA, each of it is further amplified through intermediate dsDNA templates. Eventually, NASBA yields in an exponential accumulation of RNA, more than $10^9$ fold amplification within 90 min at
The length of target sequence is restricted to approximately 100-250 base pairs (bp) to achieve an efficient amplification\(^\text{81}\). Both amplification and detection of NASBA require RNase-free conditions to prevent RNA degradation. NASBA has been used for the detection of different RNA viruses such as hepatitis C, HIV, influenza virus, Newcastle disease virus, and swine fever virus\(^\text{82,83,84}\).

**Strand Displacement Amplification (SDA):**

SDA generates a new version of DNA template to be amplified using two types of primers for each strand of target DNA. The amplification primer contains a restriction enzyme recognition site and binds to a known region of the template. The second primer binds upstream of the amplification primer in order to displace the amplification template. The displaced template then hybridizes to another opposite strand, containing the enzyme restriction site that was present in the amplification primer. Upon cleavage of the restriction site containing template, DNA polymerase recognizes and synthesizes a new strand which displaces the previous strand. This process of nicking, polymerization and displacement occur continuously for both DNA strands at an exponential rate\(^\text{85}\). SDA can generate \(10^7\) copies of target DNA in two hours at \(37^\circ\text{C}\)\(^\text{80}\). This method requires high heat \(95^\circ\text{C}\) to denature dsDNA. Exponential amplification of SDA can be performed using one amplification primer. A variety of pathogens have been detected using SDA, including HIV type 1, *M. tuberculosis*, *Chlamydia*, and *N. gonorrhoeae*\(^\text{86,87,88}\).

**Loop-mediated isothermal amplification (LAMP)**

LAMP is another isothermal, DNA amplification method that relies on a set of four to six primers, DNA polymerase with 5'-3' exonuclease and strand displacement activity. The new template for amplification is generated from target DNA using inner and outer primer pairs that form single stranded DNA with dumbbell-like loop structures. Exponential amplification occurs as a result of continuous cycles of strand displacement during elongation with the formation of new loops. LAMP is known to be highly sensitive, with as few as six copies of DNA needed to be detected in a single reaction of 30-60 min, and sensitivity can be increased using more primers\(^\text{89}\). With an additional step of reverse transcription to the LAMP assay (RT-LAMP), RNA templates can also be amplified and detected. LAMP assay has been used for various RNA viruses such as HIV, Zika, Chikungunya, Dengue and Ebola\(^\text{90,91,92,93}\).

**Helicase-Dependent Amplification (HDA)**

The HDA method, in principle, is similar to PCR as it is a three-cycle process: template separation, annealing and extension of primers. However, HDA
uses accessory proteins such as single-strand binding (SSB) protein to prevent dsDNA formation, and MutL to initiate helicase-unwinding activity. The primers are then annealed to their ssDNA target and subsequently extended with DNA polymerase. All these reactions occur in a single step with a constant temperature in a continuous cyclic fashion. Both the target and the complementary sequences are amplified. Exponential accumulation of DNA in a single reaction by HDA results in over a million-fold amplification. HDA has been applied to the detection of Herpes Simplex Virus (HSV), *M. tuberculosis* and other pathogens.

**Recombinase Polymerase Amplification (RPA)**

RPA employs recombinase for target amplification by catalyzing primer hybridization with homologous target sequences. Recombinase aids primers to the specific site, the enzyme allows for opening up of the dsDNA, and subsequently ssDNA templates are stabilized with SSB proteins. Next, primers are elongated using DNA polymerase that displaces new strands with the old ones. The *de novo* strand is used as an amplification template for the next cycle, and the cyclic repetition of the whole process results in an exponential amplification of the initial target sequence. On the contrary to other aforementioned methods, RPA can amplify long amplicons as long as 1-2 kb. RPA can generate millions of copies in approximately 40 min and thus it has high sensitivity with a limit of detection as low as a single copy. RPA also offers a wide range of operational temperatures from 25°C to 42°C. It is commercially available and has already been applied outside lab settings. RPA has been successfully applied to many important pathogens such as Rift Valley fever virus, Ebola virus, Bovine coronavirus, MERS, Dengue virus, HIV type 1 and Influenza A.

**Linear isothermal nucleic acid amplification method: Rolling Circle Amplification (RCA)**

As previously mentioned, isothermal amplification methods have several advantages, including the ability of high amplification efficiency and detection sensitivity. However, they suffer from technical complexity due to primer design and non-specific amplification due to exponential reaction kinetics. In addition, multiplexing becomes an important criterion to be considered for diagnostic applications. Isothermal amplification strategies with linear amplification kinetics have emerged to offer more convenient design and improved specificity. Rolling circle amplification (RCA) is one such simple yet efficient isothermal enzymatic method that employs unique polymerase activities on
circular templates at an ambient temperature (37°C). Among the polymerases, phi29 (Φ29) DNA polymerase is the most favorable one for RCA due to its high processivity and strand displacement activity. The polymerase activity provides continuous amplification of the circularized target, and its 3′ to 5′ proofreading activity boosts its processivity and replication fidelity. Additionally, the exonuclease activity of Φ29 DNA polymerase can digest the overhanging tail from the 3′-end of the target site, thus it can use the target as the RCA primer (generally as target primed RCA) without any need for external primers, subsequently allowing sequence specific detection.

In this thesis, I have applied padlock probe (PLP)-based isothermal RCA to develop methods for viral disease diagnostics. Therefore, the RCA methods used in the projects solely depend on padlock probe(s) and their strategy specific designs that provide versatile assays such as for detecting different viruses in the same sample mixture (multiplexing), differentiating viral RNA (vRNA) and its complementary RNA (cRNA), simultaneous detection of viral DNA and RNA in situ, and high coverage detection of variable emerging RNA virus strains.

The fundamental events required for PLP-based RCA, as seen in Figure 6, are (a) hybridization of the PLP to the target (b) ligation of the PLP by a DNA ligase and (c) amplification of the circularized PLP by Φ29 DNA polymerase. The PLP plays an essential role in RCA from the point of view of assay specificity, multiplexability and most of all, creating a circular template for the amplification. The PLP is a linear oligonucleotide composed of two target complementary arms, each of which are approximately 20 bp long and connected by a 40-50 bp long non-complementary sequence. The probe arms have a free phosphorylated (-PO₄) group at the 5′ end and a hydroxyl group (-OH) at the 3′ end, while the non-hybridizing backbone carries detectable functions. Upon hybridization of the two arms to their complementary sequences in a juxtaposed manner, probe arms wrap around due to the helical nature of DNA and topologically create a padlock structure on its target. The nick is sealed with a DNA ligase by forming a phosphodiester bond between the 5′PO₄ and 3′OH ends of the PLP arms in proximity to each other. As a consequence of hybridization and ligation events, the linear probe becomes a closed circle probe locked on its target, which then serves as a template for the amplification event. Specificity of the RCA assay relies mainly on PLP hybridization and ligation events. First, the PLPs are sequence selective, thus only target molecules with the correct base pair will be selected. Second, simultaneous hybridization of the two probe arms that will bring them into proximity is required for the ligation event to take place. Third, even if any non-specific PLP hybridization occurs, high fidelity DNA ligases such as Thermus thermophilus (Tth DNA ligase) with great discrimination ability against mismatches around the ligation junction prevents circle PLP formation and
eventually unligated circles will fail to be amplified. On the other hand, this property of the ligase can be used to distinguish among related target sequences with one base difference at the ligation site such as single nucleotide polymorphism (SNP) detection. Furthermore, this provides multiplexability and identification of nucleic acid variants in a complex mixture without allowing for any cross reactivity.

Figure 6: Padlock probe and RCA: A) Structure of a padlock probe with target-specific arms and a generic backbone comprising of sites for the binding of restriction oligonucleotides and detection oligonucleotides. B) Hybridization of probe to target at the juxtaposed target-specific arms, followed by subsequent ligation event. C) Rolling circle amplification using phi29 polymerase generating RCA products (RCPs) that collapse into micrometer-sized DNA coils. D) Labeling of RCPs using fluorophores enabling microscopic detection and digital quantification.

Finally, removing non-specifically trapped PLPs with the aid of stringent washings can further enhance the specificity of the assay. Overall, the specificity of the assay is ensured by PLP and ligation events, while the sensitivity is achieved by highly processive polymerase amplification. Owing to the high polymerization rate of phi29 DNA polymerase ($10^3$ nt per minute), a 100-nucleotide long circular template is copied several hundred times in an hour. Subsequent to amplification, RCA products (RCPs) consist of single
stranded DNA with tandem repeats which spontaneously collapse into approximately 800 nm to 1 µm size DNA coils. If these coils are labeled with fluorophores, they can be visualized as bright spots under the microscope. They can also be digitally counted in a microfluidic system coupled with confocal microscopy. Each spot exhibits a single RCA product obtained from a single molecule amplification that is tethered to the target of interest. Therefore, PLP-based RCA provides single molecule detection and also spatial information by in situ detection as the amplified product is still attached to its target DNA.

Despite the aforementioned advantages, the limit of detection (LoD) of RCA methods has been greatly limited due to its linear amplification kinetics. However, the sensitivity can be boosted in various ways, such as by increasing the number and local density (signal-to-noise ratio) of signals. For instance, using several PLPs per target gene can increase the number of signals while allowing a longer amplification time can increase the density of the signals. Moreover, coupling RCA assay with sensitive read-out platforms can also enhance the limit of detection significantly. Another way of increasing the number of signals is an additional round of amplification followed by an intermediate digestion of RCPs, which is called circle-to-circle amplification (C2CA). This method, schematically shown in Figure 7, allows for an increase in the amount of amplified product per single target to be detected. After the initial linear extension of the circular target, RCPs are fragmented with a digestion enzyme into small monomers using short oligonucleotides that are complementary to restriction sites introduced via the backbone of PLPs. Afterwards, monomers are re-circularized and ligated onto the excess undigested restriction oligos, which will serve as a template for the second round of amplification. The number of end amplification products of C2CA depends on the repetitions of the PLPs generated during the initial amplification, thus allowing longer amplification time can increase the number of repetitions and boost the overall sensitivity of the detection even further. Thanks to consecutive round(s) of amplification in C2CA, the initial single molecule detected with a 90 nt long PLP can be further copied 1,000 times more per hour, thus increasing the detection sensitivity a millionfold.
Detection of Rolling Circle Products

After the RCA, it becomes essential that the produced concatemeric RCPs are detectable by a suitable detection tool or technology, in order to estimate the outcome of the performed reaction, both qualitatively as well as quantitatively. Since RCA produces micrometer-sized DNA blob-like structures, it enables digital quantification and can be easily visualized or measured using suitable label and label-free methods. In this section, I will discuss some of these methods that have been used for the detection of RCPs (Figure 8), in turn becoming relevant for exploitation in diagnostic applications. Such methods are broadly classified as either labelled or label-free, depending on whether any additional molecular tags aiding their detection have been attached to the RCPs or whether the produced RCP molecules are measured directly in the detection platform, respectively.
Figure 8: Read-out platforms for RCP detection. The collection covers labeled and label-free methods ranging from optical and magnetic to electrical and electrochemical techniques. Commonly used PoC platforms such as lateral flow, glucose sensors and smartphone detection are also shown, alongside microfabrication and microfluidic-based methods. Images A, C, F, H, I, J, K, M reproduced with the permission from ref. 13, 130, 120, EMPE Diagnostics AB (https://www.empediagnostics.com), 140, 165, 143, 141).

Like other molecular techniques, RCA can also exploit the most commonly used optical modes of detection for visualising or quantifying the produced RCPs. Fluorescence-based detection is the most widely used method, where the RCPs are fluorophore labelled, either post-synthetically via hybridization probes, or by binding affinity probes to tagged dNTPs or directly during synthesis by incorporation of fluorescent dNTPs into the nascent strand\textsuperscript{115}. The post-synthetic fluorescence-based labelling technique has been extensively used not only for detection of pathogens\textsuperscript{116}, but also to understand the reaction mechanism and assay kinetics, including C2CA\textsuperscript{114}. Use of molecular beacons for hybridizing with RCPs, where the fluorophore and the quencher are moved away from each other in case of binding to the target causing the hairpin-shaped molecules to open thereby restoring fluorescence, have also been recorded in literature\textsuperscript{114,115}. Quantum dots are also used to substitute fluorophores for RCP detection in order to obtain a better signal-to-noise ratio owing to their increased quantum yield\textsuperscript{117}. Intercalators such as SYBR Green, that bind to double-stranded DNA molecules, are also used for RCP detection\textsuperscript{118,119}.
Many of the fluorescence-based methods for RCP detection make use of microscopy, thanks to the micrometer range of the amplicons produced by RCA\textsuperscript{111,120}. To ease the routine detection of RCPs, customized automated instruments with the desired lasers (matching the fluorescent tags used in the detection probes) can scan the input volume of the RCP solution yielding their digital counts\textsuperscript{110}. Such a digital RCP counter from Q-Linea AB (Sweden) has been extensively used for RCP quantification in various experiments reported in this thesis. Microfluidic adaptations for enriched RCP detection in a single field-of-view enhance the sensitivity and enable digital analysis in any laboratory equipped with a normal epifluorescence microscope. This technique has been demonstrated for quantification of bacteria together with antibiotic resistance markers\textsuperscript{112} as well as clinical virus samples\textsuperscript{121}. In the latter study, possibilities towards parallel sensing of different RCPs from different reactions using a microscope slide chip format have also been underlined. In this thesis, paper II and manuscript III, pertaining to poultry viruses and tropical viruses (Ebola, Zika and Dengue), respectively, use these microfluidic chips for membrane-based RCP enrichment and quantification, thus bringing RCP detection closer to field settings. Advanced microscopy methods, such as scanning electron microscopy\textsuperscript{122} and confocal laser scanning microscopy\textsuperscript{123}, have also been utilized for clearly visualizing the stretched RCPs, and atomic force microscopy\textsuperscript{124} for visualizing the topography of the RCPs on the sensor surface. Meanwhile, portable and cost-effective optical imaging designs like smartphone-based microscopy systems are in use for RCP detection and quantification\textsuperscript{120} as efficient telemedicine technology to promote on-site diagnostics\textsuperscript{125}.

Non-fluorescence based measurements such as chemiluminescence\textsuperscript{126} and bioluminescence\textsuperscript{127} have been used for the estimation of target DNA concentration. Likewise, colorimetric approaches have also been adopted for RCP detection, especially for use in resource-limited settings in order to avoid the technical complexities involved in fluorescence detection and to aid direct visual detection where possible as in the case of lateral flow nucleic acid biosensors\textsuperscript{128,129}. There is existing scope for using established PoC colorimetric units detecting biomolecules\textsuperscript{130} to be used for RCP quantification. Miniaturised microfabricated photodiodes for enhanced optical sensing have been exploited for RCP detection, with immense scope for microfluidics-compatible sensors\textsuperscript{131}. Such sensors have been used for the sensitive detection of viruses such as Ebola and Influenza. Optical sensing of RCPs has been extended to the submicron-level using nanofluidic diffraction grating for the detection of biomolecules\textsuperscript{132} as well as pathogens like TB bacteria and Human Papilloma Virus\textsuperscript{133}. 

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RCPs can also be labelled with magnetic beads enabling their detection using a suitable magnetic sensor. In this context, volume-amplified magnetic nanobead assays have been reported\textsuperscript{134,135}, also with possibilities for multiplexing\textsuperscript{136}, sensitive detection of \textit{E. coli} (as few as 50 bacteria)\textsuperscript{137}, sensitive detection of bacterial spores\textsuperscript{138} and duplex detection of \textit{Vibrio} and \textit{E. coli}\textsuperscript{139}. Superconducting Quantum Interference Devices (SQUID) that perform detection of how frequency dependent magnetic response changes in the frequency domain due to specific adsorption of biomolecules on the surface of magnetic particles have been used for quantifying the RCPs in solution\textsuperscript{140}.

When the RCPs are decorated with gold nanoparticles, relevant gold enhancement chemistry could be performed on the particles on the beads, thereby creating metallic nanowires that become electrically conductive. The resistance between the electrodes connected by such nanowires could be measured for estimating the sensitivity of the analyte concentration\textsuperscript{141}. These electrical sensors hold multifold benefits such as ease in integration, relatively low cost, potential for downscaling, ease in the signal acquisition process and processing for digital output. This in-plane nanowire formation has been further extended to out-of-plane nanowire formation with scope for applications in 3D microelectrical devices for biological sensing\textsuperscript{122}. MEMS-based (microelectromechanical systems) devices offer the advantage of real-time (online) monitoring of events occurring in biomolecular interactions, which were rightly capitalized for monitoring RCP formation. An RCA-assisted surface plasmon sensing platform has been reported for understanding the immobilization, hybridization and amplification events during RCP formation\textsuperscript{142}. Likewise, quartz crystal microbalance devices have also been used for monitoring RCP generation on sensor surfaces\textsuperscript{124,143}. Advanced sensor systems precisely defining biomolecular interactions at the nanoscale such as silicon nanowires and field effect transistors can also be used for monitoring RCP formation. All the above studies shed light upon the molecular events associated with RCP formation, thereby opening doors for utilization of relevant detection platforms compatible with the assays used.

RCA goes point-of-care

Although there are existing established molecular assays in vast numbers for biosensing and bioanalytics, the need for rapid, accurate and portable identification of pathogens is still a pressing issue in clinical diagnostics. As mentioned in section “Conventional vs. Molecular Methods for Viral Diagnostics”, conventional diagnostic protocols for identification of pathogens require large numbers of a pure cell culture, with enrichment and pre-selection steps consuming appreciable time and labour\textsuperscript{144}. Moreover, since the conventional diagnostic methods rely upon complex instrumentation, expensive reagents,
increased assay duration, electricity and skilled labour, alternate protocols become highly demanded for use in both developing as well as developed countries. To address these issues, bedside diagnostics for near-patient testing using PoC systems become relevant, especially in RLS lacking centralized laboratories for regular diagnostic testing of infectious diseases, in particular. With increasing number of biosensors for molecular diagnostics, the need for cost-effective, miniaturized and disposable test devices are on the rise, for which ‘micro total analysis systems’ (µTAS) or ‘lab-on-chips’ (LoC) have been extensively developed during the past decades. In addition to appreciable size reduction, such systems were also tuned for enhanced bioanalytical performance with improved specificity and sensitivity. Lateral flow tests, the most popular, versatile yet still simple PoC format systems, have found increased technological adaptations and applications for detecting a wide range of diseases. Such microfluidic LoC-based PoC technologies strongly promote on-site diagnosis and treatment, thanks to their simple sample-to-answer format and quick analysis time. In addition, such systems can effectively miniaturize and integrate most of the functional modules of the assays generally performed in centralized laboratories that aim to detect cells, proteins, nucleic acids or metabolites. Thus, specialized biosensors focusing on detecting specific types of biomolecules are on the rise, and in this context, DNA-based biosensors that especially make use of the self-recognition properties of DNA molecules, in a functionalized and highly controllable fashion, are gaining popularity for pathogen sensing. Along these lines, RCA also offers broader opportunities for controlled fabrication and functionalization of DNA-based biosensing protocols.

Various RCA-based biosensors have been demonstrated for their bioanalytical performance during the recent years, especially for their simple approach and rapid detection, thereby bringing RCA closer to PoC settings. An integrated simplified fluidic DNA analyzer based on magnetized bed reactors was demonstrated for biomolecule manipulation, where the efficiency of individual RCA/C2CA steps was checked for its analytical performance. A gold nanowire-based electrical detection of RCPs on a porous membrane substrate was proposed for potential multiplexed PoC biosensing. Use of clinical samples for an inexpensive digital RCA-based assay was implemented with increased specificity and multiplexing capability for both detection and subtyping of seasonal influenza. This study used the MRE method, allowing for digital quantification of RCPs, thus avoiding the need for any specialized instrumentation for detection, one of the prerequisites for PoC systems. Simple membranes become very useful in diagnostics both acting as a substrate as well as supporting units for detection, thereby simultaneously avoiding complexity and offering cost-effectiveness with required efficiency standards. Likewise, mobile phone-based on-site biomolecular analysis, promoting
cost-effective and PoC molecular diagnostics in RLS\textsuperscript{120,125,156}, as already described in section “Detection of rolling circle products”. A double amplification (C2CA) procedure combined with an electrochemical readout for yoctomole genosensing of Ebola virus cDNA resulted in a highly sensitive, rapid, inexpensive, robust and user-friendly approach finding use in primary care at RLS\textsuperscript{113}. From the technical side, strategic combinations of assay platforms such as PLP-lateral flow has been demonstrated first of its kind for the proficient detection of multi-drug-resistant \textit{Mycobacterium tuberculosis} to be performed at RLS, together with a note on the drug-susceptibility pattern to shed light onto appropriate infection control actions\textsuperscript{129}. The current thesis also focuses on similar RCA tests for PoC applications, mainly on viral diagnostics. In this context, both paper II and manuscript III discuss MRE-based simplified detection of veterinary and tropical viruses, respectively, with samples from the field, thereby bringing RCA further close to on-site diagnostics. Similarly, manuscript IV discusses a simplified biosensor-based approach for easy yet effective detection of nucleic acids from pathogens.

\section*{Microfluidics for RCA Applications}

As mentioned in the previous section, approaches towards PoC applications require distinct features such as miniaturization, integration and automation. For all the above-mentioned three characteristics, processes like microfluidics become indispensable. The same holds true for the use of microfluidics in RCA optimized towards various diagnostic and other applications. The advantages of microfluidics for these include not only practical ones like smaller reagent/sample volumes, short reaction times, and relatively low cost but also technical ones like possibility for parallel operation (multiplexing), achievement of high resolution and sensitivity, small footprints for analytical devices, etc.\textsuperscript{157,158}. Physical properties such as laminar flow, fluid resistance, molecular diffusion and surface properties define the type of applications that microfluidics can offer for bioanalytical tests\textsuperscript{159}. Exploitation of such features decide the type of materials (PDMS, glass, COC, etc.), type of samples, pre-treatment protocols, fluid actuation (pneumatic, capillary, centrifugal, electrokinetic, etc.), fluid mixing and signal detection (similar to RCP detection methods described in the previous section). All these have given rise to diverse microfluidic products for molecular diagnostics, for a variety of applications ranging from simple quantification of nucleic acids (Bioanalyzer from Agilent) and blood type identification (ABO card\textsuperscript{®} from Micronics) to complex pathogen identification such as GeneXpert System\textsuperscript{®} from Cepheid and Pima\textsuperscript{TM} CD4 Analyzer from Alere Technologies for CD4 count used in HIV diagnosis. In this section, different approaches towards microfluidic RCA implementation will be discussed which could be applied for diagnostic applications.
Thermoplastic microfluidic platform for RCA-based single molecule detection has been demonstrated with sensitive on-chip fluorescence detection on inexpensive substrates. This was based on a simple fabrication process consisting of rapid injection molding of thermoplastics, silica deposition and covalent attachment of an unstructured flexible lid, in turn offering advantages of combined elastomer technology and high-throughput Compact Disc injection molding that yield about 100 chips in a couple of hours (as compared to PDMS-based structures for the same format that might require about a week). The need for such disposable microchip integrated DNA amplification is based not only on an appreciable (and obvious) decrease in the required amount of sample and reagents, but also on a smaller heat capacity that allows rapid changes in temperature which significantly reduces the overall assay time (even for isothermal assays that lack many ramping steps) and on a highly parallel environment offered by these chips for the analysis of multiple genetic samples. After the on-chip RCP detection, chip-based electrophoretic detection of RCPs, that combined RCA with microchips using an electrophoretic port as an RCA chamber and RCP analysis by microchip electrophoresis, was shown. 25 ng of Vibrio cholerae was detected in 65 min (including microchip electrophoresis) in this study. In addition to rapid RCP detection (in a few minutes after the start of the reaction), these chips also offered about 5-fold reduction in reagent consumption. Such studies clearly open the scope for fully integrated chips that combine both assay and detection in a reasonably compatible format for bioanalytics.

One way to combine the assay and detection on-chip was shown by Sato et al., where a solid phase (bead-based) capture in microchannel was performed. This method not only enabled robust washing cycles (unlike homogeneous assays with contamination issues resulting in increased background) but also provided decreased analytical area, resulting in sensitive detection (up to 88 ng) of Salmonella enterica. Such microchips for dual-purpose (assay and detection) clearly offer long-term benefits for integration and claim user-friendliness. The next step to such a combinatorial microchip is automation, and Kuroda et al., demonstrated a microfluidics-based in situ padlock probing and RCA in a simplified automated format. The study involved counting of amplified mitochondrial DNA fragments in HeLa cells, with microfluidic conditions clearly affecting the assay efficiency. An interesting feature of this microchip is the reduced product volume (< 10µL) used for detection (this is almost three times lower than the volume used for commercial RCP counter from QLinea AB).

A rotary microfluidic device for L-RCA (ligation-rolling circle amplification) for fluorescence-based multiplex SNP typing of TP53 (tumor protein p53) gene was built with three glass layers comprising of a channel wafer, Ti/Pt electrode-patterned RTD (resistance temperature detector) wafer and a
rotary plate with 12 fabricated reaction chambers. Use of a rotary plate eliminates the need for microvalves and micropumps to control channel flow, thus offering simplicity and portability to the system. Next level of nanofluidic channels for RCA-based assays were demonstrated for label-free detection using nanofluidic diffraction gratings combined with photodiode-based detection for the identification of Human Papilloma Virus and Tuberculosis bacteria genes. Automation of such an RCA-based system has been demonstrated on a digital microfluidic platform for the detection of bacterial DNA in the attomolar-range. Towards simplification and multiplexing, a microfluidic magnetized bed reactor chip was developed with a single chamber combining fluidized bed and polymer-based microarray read-out. The efficiencies of individual RCA and C2CA steps on such platforms proves to be promising for use in automated assay system for a wide range of application including diagnostics. Recently, a sensitive and an inexpensive RCA-based digital DNA analysis by microfluidic enrichment of RCP molecules has been demonstrated, together with multiplexing features. This novel digital analysis strategy allowing single molecule counting with conventional low magnification fluorescence microscopy has been adapted to detect clinical influenza samples, and also adapted in the current thesis for poultry and tropical virus detection (as discussed in paper II and manuscript III).

In brief, the advantages offered by microfluidics can go a long way in allowing fast dissemination of test results in remote regions and RLS. Relevant state-of-the-art technologies in microfluidics have already been extensively used for the detection of infectious diseases, but still offer new opportunities by providing the right scope for development of the associated technologies to their fullest potential, thereby creating business models for the commercialisation and deployment of diagnostic protocols and products.
Present Investigations

I. Simultaneous Single-Cell In Situ Analysis of Human Adenovirus Type 5 DNA and mRNA Expression Patterns in Lytic and Persistent Infection

Human adenoviruses (HAdV) are one of the most common dsDNA pathogen infecting humans. To date, over 60 different human adenovirus serotypes have been designated into seven subgroups\textsuperscript{168}. Different serotypes can infect a variety of cell types, thus causing a broad spectrum of diseases such as conjunctivitis, gastroenteritis, hepatitis, myocarditis and pneumonia. Recently, fatal infections associated with newly emerged adenovirus types have been also reported\textsuperscript{169,170}. Depending on the type of cell and replication cycle, adenovirus may cause acute or chronic infectious diseases. Interestingly, adenovirus type C exhibits both lytic and latent type of viral life cycle upon their infection of epithelial cells and lymphocytes, respectively. The former, which is the most typical life cycle of adenovirus, releases viral particles in several hours (24-72h) by lysing cells. However, in the latter, the virus genome is maintained in a quiet state for longer period of time (up to months) and can be reactivated by unknown mechanisms. Human type C has been shown to be the most prevalent adenovirus detected in surgically removed human tonsils and adenoids. High accumulation of viral DNA and mRNAs occurs with the onset of viral replication during a lytic infection and viral genome do not correlate well with the amount of viral mRNAs due to silenced transcription. Most of the current methods to study adenovirus DNA content and RNA expression rely on cell population studies, which neglect the fact that individual infected cells exhibit viral genomics and transcriptomics heterogeneity. Hence, studying different mode of infections at the single cell resolution can elucidate cell-to-cell variations and provide a better understanding of their infection characteristics.

We developed an \textit{in situ} PLP-based RCA method, which enabled a simultaneous detection and quantification of HAdV-5 DNA and its various encoded mRNAs in individual cells of a heterogeneous cell population. We then designed padlock probes targeting viral DNA as well as viral mRNAs (early and late mRNAs) and monitored their accumulations in a spatiotemporal manner. To achieve this, combinations of enzymatic steps were applied to make targets available for padlock probe recognition. The double stranded viral DNA was
fragmented and recessed sequentially using restriction and digestion enzymes in order to generate ssDNA, while viral mRNAs were reverse transcribed to cDNA. In particular, the ligation sites of PLPs were positioned at exon-exon junctions of viral mRNA targets in order to discriminate splice variants and to prevent cross-reactivity with viral DNA target. We compared temporal changes of viral replication and expression patterns in individual infected cells, both in lytic and latent mode of infection. Our single cell quantification analysis that was applied to lytic infected cells indicated heterogeneous cell subpopulations with distinct characteristics defined by viral DNA contents and mRNA expression patterns. We also studied HAdV-5 long-term infection. For this, BJAB cells were infected with HAdV-5 for six days and the method was applied. With our PLP, viral DNA and mRNAs were detectable in the BJAB cells. Single cell quantification allowed grouping of infected cells into three clusters based on their viral content and mRNA expression levels. The majority of infected BJAB cells exhibited some levels of viral mRNA with low or no viral DNA content, which was interestingly contradicting to the late stage of lytic infection (25hpi) where majority of cells had high viral content. On the other hand, only few cells showed certain similarities to 25 hpi of lytic cells; high levels of viral DNA with low expressed mRNAs. Our method revealed, for the first time, different accumulation pattern of splice variants of the early viral transcripts depending on viral DNA content of individual infected cells.

Several methods have been employed for in situ HAdV-5 DNA and mRNA detection. Immunostaining of viral proteins that are associated with viral DNA during its replication phase has been successfully used for visualization of the replication centers in situ. Moreover, in situ hybridization of fluorescently labelled probes targeting viral DNA has also been shown. In situ adenovirus mRNA detection was accomplished using fluorophore labelled probes. However, simultaneous in situ detection of adenovirus DNA and mRNA has not been shown so far. In addition to in situ detection, quantification of adenovirus nucleic acids has been achieved using qPCR; however, it relies on averaging infected cell populations, thus masking population heterogeneity. Our versatile padlock probe-based method along with the single cell quantification analysis allowed simultaneous detection of viral DNA and mRNA in both short- and long-term infection. This method further allowed the characterization of the molecular details during different time course of infection as well as different mode of infection (lytic and persistent).
II. A novel mutation tolerant padlock probe design for multiplexed detection of hypervariable RNA viruses

Infectious diseases can be caused by pathogens, including bacteria, fungi, parasites or viruses. Especially viruses demand high attention, and in particular, the subgroup of RNA viruses is known to be hypervariable due to high mutation rates that leads to emerging and re-emerging outbreaks. Classical approaches for detection of viruses are virus isolation and serology, which are time consuming and often lack of sensitivity. Molecular assays such as PCR have become the method of choice owing to their outstanding sensitivity, specificity for pathogen detection and turn-around time. However, for hypervariable viruses, such as Newcastle Disease Virus (NDV), PCR-based assays require continuous assay validation and optimization to reliably detect all relevant newly emerging NDV classes. In this context, next-generation sequencing technologies could provide the means to overcome these limitations. However, their use in early epidemic control settings remains challenging due to practical implementation and assay output parameters.

The PLP-based assay presented in this paper tackles the current need for a robust and reliable detection of rapidly evolving RNA viruses due to high mutation rate, with using poultry virus, NDV, as a model RNA virus. PLPs were designed based on an in silico scoring system which was performed by first constructing a phylogenetic tree from 335 NDV sequences and dividing them into two subgroups, A and B. Next, sequences were scored in terms of homology to achieve high coverage as well as mutation tolerance by introducing degenerative bases. Furthermore, the rationale PLP design took the DNA ligase footprint into account in order to achieve high specificity and allowing NDV group differentiation. We confirmed high specificity of the rationale PLP design by detecting NDV, alongside other poultry viruses, namely Infectious Bronchitis Virus (IBV) and Avian Influenza Virus (AIV) from virus isolates with high accuracy; this was done mainly considering multiplexing, a key parameter for parallel detection of several pathogens. We achieved multiplexed detection by introducing virus-specific barcode sequences in the PLP backbone. Furthermore, adapting recently established MRE strategy enabled us to detect less than a dozen of RNA copies in a multiplexed manner.

In conclusion, this approach addresses the current need for tolerating hypervariable RNA virus detection in a multiplexed format with the potential of
being adapted for the diagnosis of viruses infecting humans. Such an approach is discussed in the following work for the detection of tropical virus infection of humans.

**III. Multiplexed rolling circle amplification detection of Ebola, Zika and Dengue towards point-of-care diagnostics**

With a transition from highly mutative poultry viruses in the previous manuscript to infectious diseases in humans in the current one, as they are spreading faster than ever in human history due to the growing population and globalization. Although hygiene and healthcare improved significantly in the recent century, infectious diseases still pose a major burden to public health and global economy. Today, they account for more than 25% of the annual deaths worldwide with a clear upward tendency. Especially developing countries suffer from high mortality as hygiene and medical infrastructures are rudimentary or non-existent. Another factor contributing to the high infection rate in these countries is the close contact of humans and animals as approximately 75% of emerging pathogens are zoonotic, thus, being a direct result of this close communication; examples include, HIV, avian influenza and Ebola. Especially, the latter caused recent epidemics in West Africa and showed the need for new diagnostic solutions and disease control. Current diagnoses are based on serology, antigen and molecular tests with techniques such as electron microscopy, ELISA and RT-PCR which require complex protocols and trained personnel, thus limiting their use in RLS.

Towards achieving decentralized healthcare, we propose a PLP-based assay combined with a new version of the simple pump-free RCP enrichment strategy from paper II as an alternative method for current viral diagnosis. For this, we initially demonstrate a PLP-based single RCA method, for the detection of EBOV from cell culture isolates as well as clinical samples. Furthermore, we propose a new PLP design by targeting not only cRNA (complementary RNA) but also vRNA (genomic RNA) that has the advantage of not only increasing diagnostic sensitivity but also enabling the understanding of molecular pathogenesis. Towards parallel detection of pathogens, we developed a multiplexed RCA-based assay for the detection of tropical viruses comprising of Ebola, Zika and Dengue, thus aiding differential diagnosis of Ebola Virus Disease. The multiplexability of this approach was successfully demonstrated on cell culture isolates by confirming minimal cross-reactivity between the three mega cocktail PLP systems (mixture of ebola, zika and dengue PLPs). The assay sensitivity was confirmed by running the assay on Ebola
positive and negative patient samples. This further underlined the specificity as some of the Ebola negative patients were Malaria positive. To further increase the assay sensitivity and allow early virus diagnosis, we adapted the MRE strategy as presented in paper II towards the needs for RLS. For this, we custom-designed a new, pump-free version of the enrichment chip to allow for equipment-free RCP trapping. Immediate next steps include the demonstration of the performance of the new enrichment chip in terms of sensitivity and ease-of-use, and its validation on patient samples.

This work provides evidence that the developed RCA-based method could potentially be an alternative to conventional molecular tests towards decentralization of healthcare and improved patient care in RLS for viral diagnostics.

IV. The sweet detection of rolling circle amplification: Glucose-based electrochemical detection of virus nucleic acid

As discussed in previous sections, NAATs are used for reliable and rapid diagnostics to tackle the constant threat of emerging and re-emerging infectious diseases. This is mainly due to their feature of being highly sensitive and specific at the same time when compared to classical methods. However, most of these instruments can only be found in centralized healthcare centers for routine diagnostics and thus excluding third-world countries which lack such medical infrastructures. The use of isothermal amplification methods has the potential to bring NAATs to the PoC as technical requirements for those assays are greatly reduced. Nevertheless, one of the main challenges remains as the read-out that requires sophisticated and bulky optical setups most often. Due to the vast advances in semiconductor technology, devices such as photodiodes and field-effect transistors have found their way into biotechnology and modern diagnostics. However, even such advancements that depend on sophisticated instrumentation and facilities (such as cleanroom) for fabrication and measurement need to be simplified, bringing inventions in molecular diagnostics very close to bedside detection.

In this manuscript, we aim to provide an alternative approach for conventional fluorescence-based detection by adapting solid-phase RCA on beads to electrochemical detection of RCPs generated from viral RNA. For this, we use screen-printed electrodes which are very appealing for diagnostic purposes as they are extremely cheap, sensitive and small in size. Furthermore, we aim on reducing the assay costs by replacing expensive and light-sensitive fluorophores with an enzymatic labelling. Glucose oxidase is known for its good stability and widely used in the field for bedside biosensing, especially for
monitoring diabetes patients. However, the use of such a cheap and simple system can be extended towards infectious diseases for RLS. In our approach, on-bead generated RCPs are labelled with glucose oxidase to subsequently generate an amperometric signal due to the conversion of glucose to hydrogen peroxide and gluconic acid, with release of electrons. We characterized the surface of the RCPs on-beads by scanning electron microscopy and atomic force microscopy. Furthermore, the current results indicate that the sensor response depends on parameters, such as ionic strength, temperature, target DNA concentration and mode of detection (on-chip/off-chip).

By combining isothermal amplification with screen-printed electrodes, we demonstrate the proof-of-concept for a simple, cheap and miniaturized assay towards potential qualitative molecular diagnostics of infectious diseases in RLS.
Conclusion and Future Investigations

This thesis has provided an overview of the development of molecular diagnostics with the main focus on infectious diseases. The historical background of infectious diseases intertwined with the early history of humans has been described on selected examples, thereby pointing out their significant impact on ancient and modern civilisations. Although the understanding of pathogens and the importance of hygiene have significantly increased during the last centuries, pathogens still pose a greater threat to global society as well as economy. Especially the 20th century has made great progress in biomedical research due to the invention/introduction of vaccines and antibiotics. However, the emergence and re-emergence of infectious diseases makes them a constant threat, thus leading to a continuous race with science. In this context, RNA viruses show the highest emergence rate due to their highly variable genome which makes their detection challenging. Conventional diagnostic methods that are based on the direct or indirect visualisation of pathogens by microscopy and immunological methods often suffer from poor sensitivity and specificity in the case of genome shuffling/mutations, respectively. As discussed, due to these limitations, NAATs have become the golden standard for many infectious disease diagnoses owing to their high sensitivity, specificity and diagnostic conclusiveness. Among the existing NAATs, PCR has proven outstanding performance for detection of pathogens. However, it still poses complexity for multiplexing applications and is labour intensive in terms of sample preparation and the need for lab-scale instruments. Therefore, in this thesis a variety of approaches has been presented that demonstrates superior characteristics to current diagnostics such as multiplexability, sensitivity, specificity as well as mutation tolerance in hypervariable virus genomes. The methods developed in this thesis takes into account the molecular virology (Paper I), rationale PLP design (Paper II), assay simplification (Manuscript III) and miniaturisation of the read-out/detection towards PoC (Manuscript IV), in order to address some of the current limitations.
In paper I, we developed a novel PLP based rolling circle amplification technique that allows simultaneous detection of adenovirus type 5 (HAdV-5) DNA and virus encoded mRNAs at a single cell level in situ. This technique was successfully applied for detection and quantification of viral DNA and RNA in the nucleus and cytoplasm of productively and persistently infected cells. Furthermore, we characterized adenovirus life cycle in heterogeneous infected cell populations. Beyond the technical achievement of the method, we also provided novel results such as intense localization of E1A mRNAs within the nucleus of infected cells, while it was shown elsewhere to be cytoplasmic in transfected cells. We also showed another interesting biological phenomenon about the accumulation of viral early mRNA splice variants E12S and 13S related to viral DNA content at different times of infection. We also investigated histone-like viral core protein namely pVII which is known to bind to the viral genome at early phase of infection to protect it against DNA damage. Our hypothesis about the low viral DNA detection sensitivity by our method at early phase of infection was tested with cell lines expressing pVII protein endogenously. Indeed, as shown in figure 8, the overexpressed pVII in infected cells reduced the detected viral signals by RCA. Thus, we concluded that the accessibility of targets for padlock probes were hindered by pVII protein. Finally, our single cell quantification approach allowed us to group infected B cells (BJAB) into three clusters; uninfected cells, semi-infected cells and highly infected cells based on their viral DNA and mRNA contents. Thus, we showed the versatility of PLPs for in situ detection of viral DNA and mRNA concordantly without any cross-reactivity problem. This technique can be further combined with protein analyses since the method does not require any protein digestion steps. Most importantly, our technique can also be used as a clinical diagnostic tool for adenovirus detection. This could be important particularly for adenoviruses causing persistent infection in human tonsils and adenoids which of their diagnostics difficult with other conventional methods.

In paper II, a rationale PLP design was presented for tackling the current need for mutation-tolerant assays by introducing degenerated bases in the PLP based on an in silico scoring system. The scoring system took not only sequence homology into account but also the ligase footprint for maximal specificity. This concept allowed us to specifically detect NDV, IBV and AIV with high sensitivity and also in a multiplex manner. Manuscript III uses an alternative PLP design approach but with the main focus of adapting single RCA for third world countries to detect neglected tropical viruses by adapting a new pump-free design for RCP enrichment. Such a simple tool has the potential to improve healthcare in RLS as it provides high sensitivity without the need for high-resolution optics as with conventional NAATs. In brief, manuscript IV addresses the need for miniaturized read-out systems for enabling portable diagnostics needed at the PoC.
Future investigations and follow up studies include the simplification of the developed assay schemes. From the technical aspects, for RNA viruses, the use of direct RNA detection approaches could become beneficial\textsuperscript{175–178}, as the reverse transcription step requires relatively high temperatures. Such an approach could readily be implemented into the presented manuscripts on RNA viruses as well as being adapted to paper I for \textit{in situ} studies including virology but also other areas such as cancer diagnostics. The rationale PLP design presented in paper II allows easy translation towards direct RNA detection for other highly variable viruses, such as Hepatitis C, SARS or human immunodeficiency virus (HIV). Towards this, other multiplexing solutions than fluorophore-encoded RCPs are needed for RCA, such as the fluidized bed reactors\textsuperscript{154} which would also allow assay automation.

The approach used in manuscript III demonstrated the use of simple microfluidics for a vertical-flow system for RCP enrichment in combination with a multiplexed PLP design. Towards PoC applications, this could be combined with dried assay reagents and solid-phase RCA on chip\textsuperscript{175}, thus paving the way towards instrument-free, ready-to-use diagnostics for RLS. In this context, smartphone microscopy has high potential in revolutionizing molecular diagnostics as they are portable, cheap and offer great internal as well as external features, such as high-resolution cameras and power for heating elements, respectively\textsuperscript{125}. Kühnemund \textit{et al.} demonstrated the suitability for smartphone microscopy with \textit{in situ} mutation analysis which can easily be translated to infectious disease diagnostics\textsuperscript{120}. Other portable optical solutions include the use of DVD platforms\textsuperscript{130} and photodiodes\textsuperscript{131} that could enable PoC testing in RLS.

As much as semiconductor technology has driven the development of smartphones, the same applies to electrochemical sensors which are used in manuscript IV towards miniaturization. Another advantage of such an approach is the price which allows for single-use/disposable chips, thereby reducing the necessity of complex washing protocols. A possible design extension of this approach could be to make use of the concatemeric structure of generated RCPs in combination with DNAzyme to generate an amperometric signal\textsuperscript{180,181}.

The research described in this thesis highlights significant aspects of improving molecular diagnostics for infectious diseases as it takes into account virology, assay design, assay simplification and read-out miniaturization. An integrated set-up that strategically combines all these individual modules in a miniaturised, microfluidic, multiplexable (MMM) format would be the ideal dream to push forward the future of much envisaged PoC-RCA sensor (as illustrated in Figure 9). The investigations may lead to further advancements in
the field of biomedical research towards cheaper, simpler and ready-to-use diagnostics and the potential improvement in healthcare for third-world countries.

Figure 9: Future perspective of an ideal PoC-RCA sensor coupled to a smart watch. The biosensor comprises of a ‘sample collection port’ for the loading and preparation of the sample, microfluidic transport system, an isothermal assay chamber for performing RCA, and an integration module with electrodes interfacing to the smart watch readout, all encompassed in a USB-sized device. The smart watch readout entitles easy-to-operate and ready-to-access data collection, analysis and storage, thus bringing biomedical analytics closer to clinical as well as PoC applications.

I den första delstudien undersöktes hur Adenovirusets olika former av genetiskt material (DNA och RNA) förändras under olika stadien av infektionen. Den andra studien syftade till att utveckla en strategi för att designa tester som kan detektera hypervaribla virus, exemplifierat av virus som infekterar fågelbesättningar. I den tredje studien utvecklades ett engångschip för detektion samt identifiering av ett antal tropiska virus (Ebola, Zika och Dengue). I den sista studien kombinerades glukossensorteknologi med den redan etablerade metoden för att detektera nukleinsyra (DNA/RNA), för att utveckla en Point-of-Care diagnostisk metod för virusinfektioner. Sammantaget kan resultaten i
avhandlingen leda till nya, effektivare och kompакta diagnostikverktyg för tillämpning *Point-of Care* i resursbegränsade omständigheter.
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‘Thank you’ is the best prayer that anyone could say – I say that one a lot! Thank you expresses extreme gratitude, humility and understanding’ – and, here I take the best opportunity to translate these words of Alice Walker into action: a possibility to remember with fun and frolic, each and every one I encountered during my PhD journey – my mentors, colleagues, friends, family and everyone possible! Here we go….

First and foremost, I would like to thank my supervisor Prof. Mats Nilsson for offering me this wonderful opportunity as a PhD student. It has been a great experience to be part of your research team. Your leadership fascinated and motivated me to do good research. Thank you for being always available despite your busy schedule, listening to my problems and finding solutions, being encouraging and supportive, most importantly always being cheerful and standing as a backbone, socializing well in order to make me feel comfortable to approach you easily, being very optimistic and making me believe things will work good. I was always very fascinated by your personal and scientific attitude, and you will be my role model for my future careers. I am so grateful to you for giving me the freedom to follow my ideas and passion which I personally appreciate a lot. I could not have imagined having a better supervisor for my PhD study. I have always dreamt of RCA being a super star method for infectious disease diagnostics and I am hoping that I have paved the way for it. ‘Tusen tack för allt Mats’!

My PhD journey has truly been a life-changing experience that would not have been possible without the support and guidance of many people. I truly believe people don’t meet in life without a reason. Every single person that I have met so far has given me great guidance, life experiences and contributed in different ways that made me into who I am today. More prominently during my PhD studies, I had great opportunities to meet people who had something to offer that I was lacking in terms of experience and this has helped me to improve myself and grow better as a person. Also, the members of MolDia group have contributed immensely to my personal and professional growth during my PhD journey.

MolDia is one of the groups that consists of amazing people which transformed and shaped me in all ways. I often found myself thinking ‘oh wow
this person ‘X’ is super good at doing things and I have always dreamt of being like him/her”. I have observed these situations so many times and tried to apply/mimic for my work. I think this is why our group is amazing! Mats is super good at recruiting unique people not only in terms of smartness but also with varied strengths and backgrounds which helped me to learn a lot and to collaborate in a very efficient way. Each of you had something unique that I was looking for, thanks a lot!

I would like to dedicate the following quote from the musical play “For Good by Wicked” to each and every one I have met during my PhD journey.

"I've heard it said,  
That people come into our lives  
For a reason  
Bringing something we must learn.  
And we are led to those  
Who help us most to grow if we let them.  
And we help them in return.  
Well, I don't know if I believe that's true  
But I know I'm who I am today  
Because I knew you...  
Who can say if I've been changed for the better  
But because I knew you.  
I have been changed for good...

So, let me say before we part:  
So much of me  
Is made of what I learned from you.  
You'll be with me  
Like a handprint on my heart.  
And now whatever way our stories end  
I know you have rewritten mine  
By being my friend."

To all current and previous members of MolDia, thank you very much for always being there whenever I needed help. ‘Sharing is caring’ and I observed this attitude with each and everyone in the group. Every one of you have inspired and motivated me in various ways. I enjoyed working with you and being part of this team! You guys are very smart, full of fun and great problem solvers. I have always felt very comfortable asking for any advice or help. Especially, towards the end of my PhD journey, your motivational talks encouraged me, gave me further strength to reduce my stress and to meet my deadlines.
I would like to start giving my special acknowledgments to the postdoc ladies in the group - Jessica, Carina and Navya. Thank you for providing cheerful mornings every day. I enjoyed listening to funny stories and adventures about your kids and Carina – your dog every morning 😊 It was amazing to hear how similar they were. The room was so silent and boring without your laughs! Jessica, you made me realize one can be an excellent mother and researcher at the same time. I can imagine you must be very productive in terms of managing kids at home as well as research at the same time, way too impressive and I admire that a lot! Thank you for sharing nice stories and all the help! Thank you for being a helping hand with Swedish related issues and for practicing Swedish with me 😊 Carina, apart from your passion for science, I really appreciate your love and care towards your dog! Thank you for all the nice times we had together. Navya, my Uppsala pendeltåg-mate 😊 I really missed those times, our talks in the train, going through all the troubles with the trains during winter and running from Scilifelab to catch the train and our record was 10 min, we should be in Guinness book of world records! 😊 It was a great companionship, thank you Navya! I appreciate all your help for the Ebola project. You are one of those victims who went crazy in that project. But finally, it worked!

Tom, the first person who welcomed me to the group and thought me ‘what is RCA’! I cannot express my gratitude for all the help that you offered me. Thank you for everything! I have learned a lot from you. It was an excellent collaboration on the adenovirus paper and I thoroughly enjoyed the intellectual discussions. You are very straight forward, clear headed and goal oriented and this impressed me a lot. Apart from all these, I will always remember you for your super-fast speech 😊

Marcus, I have always admired the way you handle all the ‘super cool’ projects! You get to learn cool stuff about brain every single day and I can see the enthusiasm in your eyes when you share them with us! I appreciated your challenging questions and comments. Thank you for being very friendly and nice, and mocking Nara all the time and making him ‘move’ around 😊 I enjoyed watching this! I have laughed so much when you tried explaining yourself after every joke that you made 😊 And thank you for allowing me to use your desk sometimes. Speaking of laughing, I know someone who has the most beautiful one! Anne! You are the most cheerful person I have met and always full of energy! Laugh like Anne, that’s the key 😊 I really enjoyed the time when you helped me fixing my room furniture, without you it would have been just impossible! So, thank you very very much for making it pretty! I will miss your beautiful smiles… Luckily we have got someone whose laughter is equally contagious filled with amazing jokes and a cricket master, Kamila Klemecka! 😊 Kamila, it was very nice working with you and hearing your jokes even though I don’t understand at times (: I truly appreciate all your
encouragements and motivational talks! I also admire your curiosity about other cultures. Thank you for being there.

Elin, when I first came to the group I thought you were a postdoc, not because you look like one but you looked so professional, very independent and wise. Yes, indeed you are! I am so impressed with your planning skills! Everybody can be good at planning but only few manage to make it happen. Be like Elin when planning! I was also impressed how organized your folders were 😊 Thank you helping me out with RNA and topology related questions!

Thomas Hauling, the guy who is in love with baklava 😊 and kebap! In situ sequencing and microscope master! Thank you for helping me fix all the microscope problems before I went crazy 😊

Pavan, the most polite, kind and humble person ever! Thank you very much Pavan for your kindness! I admired how you went through hectic times but you always had a smile on your face and stayed calm!

JP 😊! Thank you for all the joyful moments we shared and thanks for always being there with Elvira for any help!

Anja, I don’t know how many times I opened and read your thesis! It helped a lot, thank you!

David Herthnek, it was a great opportunity to collaborate with you on the same projects. Thank you very much for all the input, the discussions we had and for being always approachable.

Malte, thank you for the “membrane enrichment”! It just saved my blobs 😊 Your guidance has helped me a lot in the times I didn’t see a clear path in my projects.

Erik, Lord of the ring! 😊 I was so happy when you joined the group, because we finally got a native English-speaking PhD student 😊 it is also nice to have someone around who sings and whistles ;) I am looking forward to seeing RCA in organoids in the near future ;)

Olga, I truly enjoyed our doggy talks 😊 Thank you for making my days “cute” with your dog pictures!

Daniel, you have the best keyboard in the world; shiny and noisy 😊 I can accept it as a gift if you are obliged to give away due to annoyed neighbors around you 😊 😊 . Thank you for being our Herr Nilsson and Pippi’s new caretaker and bringing us your home-made ‘super gott fika’!
Di, I really enjoyed our discussions on how to make diagnostics simple and cheap! We shared so many cool ideas and hopefully we will make them real one day. Don’t eat too many hamburgers in the mornings otherwise we will be forced to invent one diagnostic tool for you! haha 😊 Thank you for the company during weekends, late hours in the lab and sharing your fruits with me!

Speaking of being in the lab all the time, Xiaoyan 😊, thank you for your company and laughs, interesting reactions to weird situations and solving all the technology problems! Your data always looks amazing, so colorful and complicated 😊 and also thank you for being my supply center 😊 whatever I need you always had it in your magic cupboard!

Chenglin, thank you for being a nice office neighbor and tolerating all the mess around. I enjoyed our history and politics discussions. At the end of the day we have the same ancestors and all politicians are crap, right? 😊

Chika, you are like an elder sister to me! You took care of me not only when I was at work but also outside the lab. Thank you for driving me home during late hours and helping me move (even though we always lost our way it was fun anyway 😊). Your mobility in the lab always impresses me, one second here next second in a different spot. I have at times thought there are two Chika’s in the group 😊. Thanks for bringing order in our lab and lab-life! I am so grateful to you for all the support you have given me! Arigatou (I hope it is the polite way).

Kae, thank you for being so kind and making our home-made buffers! And, thank you for the weekend violin concerts in the lab 😊

Ivan, thank you for all the expertise and help related to RCA, C2CA, Aquila, probe design, cutting short the sentences from manuscripts 😊, guiding me through these hectic PhD formalities. Thank you for finding oligos from nowhere, you are an oligo-finder expert! Note: Be calm like Ivan 😊

Eva, thank you for providing me your place to stay during emergency times 😊 and not getting mad after messing up with your posters 😊

Anastasia, we will be good ‘fili’ as we have a lot in common, RCA, dog, fish ;) I am looking forward to having Greek-Turkish-Spanish fikas at your super entertaining house 😊 and Alberto, last minute, still a great help with my thesis cover design. Thank you for making it exactly how I imagined! And now I know whom to ask about aquarium-related stuff 😊
Flor! What’s up! Sibel: I need help! 😊 Thank you, Flor, for your prompt response and quick reaction to my problems. You came exactly at the right time especially when I was so desperately looking for Zika 😊. You have been an amazing lab-manager, providing us with very interesting and useful tools for the group! Thank you for bringing colors and energy to our group, making our stationary zone very interesting (it was too boring before!). Many thanks for proof-reading my thesis!

Tanvir, you have been a great support for me! I have gotten not only an expert statistician friend but also a brother who is always concerned about me, making sure that I am doing alright! Thank you for all the wise talks!

Amitha, I got Indian brother (Nara) but was yearning for an Indian sister too! Here you are! You are my last minute lack in the group 😊 Thank you very much for proof-reading my thesis! For the company discovering Indian restaurants in Stockholm, vetekatten fika times and shopping (last two did not happen yet due to my hectic deadlines but will happen from now on then hopefully) 😊 I hope I can acquire your calmness by hanging out with you and hope that it will not happen the other way around hahah 😊

I would also like to thank the students, Jacob and Lukas who joined my projects and had great contributions. Isra, seninle tanıştığımıza çok memnun oldum! Türkçen benimkine bin basar 😊 Herşey için çok teşekkür ederim İsracım, canım arkadaşım.

I express my special thanks to all my project collaborators outside MolDia, who have contributed in numerous ways starting from discussion of concepts and ideas, running through the provision of samples and technology, until making relevant platforms available for my readout. Thanks to Tanel for his time and efforts throughout my Uppsala time, especially in the Adenovirus project. I also thank the collaborators from BioBridges – Sandor and Alia, as well as from EbolaMoDRAD – Ali, Cristiano, Samir and Sofia. My thanks also to Gaston and Marc for providing me space and technology for implementing my glucose sensing dreams.

I take this opportunity to thank our group neighbors – the whole group of Adnane – mainly for tolerating the noise during my discussion sessions with Nara in his place. Thanks Tim, for giving me company during my long stays, including late hours and weekends – you were always working in the lab around, eliminating me from lonely depression here! Thanks to each and everyone else in the group. And, the neighbors tolerating my discussion with Mats – the group from Christos – Alex, Akis, Andrea and Hong-Phoung, for your presence and nice talks!
Let me take this opportunity to thank a special group, close to us, both in location and action: Aman and his quasi-Indian group!! Aman – thanks for your constant support, smiles and enthusiasm, your lab space, and your excellent lectures that I always enjoyed! Thank you Sharath, you are the funniest but I enjoy your jokes a lot! I hate your t-shirts with horrible looking images but it looks nice on you, if you believe:P Anyways, Thank you for making me laugh loud always. You and Felix together are a “chatterbox” forever. Thank you, Indra, for your support with the platform for precipitation assays; thank you Ahmed for your disc fabrication and amazing drawing; thank you Tharagan for your smiles, and a special sorry for mixing up your name with that of terrible Sharath’s 😊; thank you Ruben and Ines for your presence here in Sweden and the photodiodes paper!

A special note of thanks to Adil, not only for the scientific advice but mainly for the wonderful Turkish feelings you provided with your presence. I admire your energy levels and your passion for science. Özlem, thank you for amazing talks that are full of energy and advise. I just loved it so much!

Omar, I enjoyed sharing creative scientific ideas and also about my dream cappuccino shop! Thank you for listening to all of them and spending time for brain storming! Very nice of you!

Indian Thomas, 😊 this is how we always call you! Don’t change conditions! Come on time! You are late!.... You are very enthusiastic in building amazing tools and having fun with them 😊 You are a mobile resource limited setter!

Special friends who have touched my life miraculously Alina! My Ukranian sister, we went through so many troubles together side by side, we shared so much and our hearts attached hopefully forever! I love you so much! Thank you for truly being there all the time, trying to make me happy with your surprises! You taught me how to be content and strong in the worse times ;) Thank you hosting me in your cozy place and taking care of me in the most difficult times! Katarina, thank you for all the enormous help and very wise advices! I have been enjoying a lot our mind-blowing philosophical discussions, educative talks and making dreams about future. I admire your last minute decision making ability 😊 Rageia and Tagrid! Our friendship is based on so many coincidences 😊 but I loved it! Rageia, thank you for your company, I enjoyed a lot walking around Stockholm with you;) Naify, thank you for cooking awesome Lebanese food for me and for your great hospitality.

Felix and Nara, I can’t express my gratitude to you guys! You both were not only colleagues to me but amazing friends! We created an amazing group together. I enjoyed so so so much working with you side by side. You also went through all the hectic moments together with me. Thank you for backing me
up, being extremely gentle, kind and tolerant. You guys were my biggest luck; your friendship is the best outcome of this PhD. Thank you for supporting me, encouraging me and calming me down, thank you for fighting with me in the projects and making them work after all life-long mind blowing discussions.

**Felix**, it was an awesome collaboration and experience working with you in the projects. RCA after all is not so easy but I agree it works 😊 Thank you for making me believe in “It’s OK” 😊 Zibel is so grateful for everything you have done!!!!

**Nara** aaaaaaa!!!!!! There is so much to write about you Nara! You are my supervisor, my science-guru, brother, friend…. You have taught me a lot! a lot! How to talk science, how to think and approach scientifically, how to multi task (ok, I am not good at it still but it is OK 😊 ), how to create ideas… It has been such a privilege being your student and I am so grateful for it! Thank you for storing my stuff in your storage room forever, sharing your amazing indian food, listening to me with great patience and tolerance and always ‘smiling’. I admire your great knowledge, experience and compassion that you share with others. Thank you for listening to my ideas and also making them real! As a scientist, you are a role model to me. Eat sweet like Nara to become a sweet scientist like Nara 😊. So, THANK YOU VERY MUCH NARA! Of course, there is always a super woman behind a successful man! Do you think you could do all these without Sudha? 😊 Sudha, thank you very much being so tolerant, understanding and caring! You guys are so nice and both of you transformed my life deeply! You are an awesome mother, wife, scientist and friend! Chello! Thank you for creating problems and making Nara go crazy 😊 We all love you so much!

**Daily chat between Nara, Sibel and Felix:**

*Sibel:* I just have one day left to meet the deadline and nothing is working Naraaaaa, it’s horrible!!

*Felix:* It’s ok. It’s RCA, It works. It’s easy.

*Nara:* Quatsch! Kill Sudha! Damn kid! I have a meeting now!

*End of conversation*

Canım babacım, annecim ve kardeşim. Herbirinizin emeği çok büyük ve kıymetli. Bu uzun zorlu eğitim yolculuğumda beni madden en önemli de mânen desteklidiğiniz için çok teşekkür ederim. Sizin gibi bir ailem olduğu için çok şanslıyım! Beni her zaman hayallerimin peşinden gitmek için desteklediniz, çok emek sarfettiniz. Babacım, senin gibi bir babam olduğu için gurur duyuyorum! Kendi çalışma azminle bana hep örnek oldun. Desteğin için çok teşekkür ederim. Annecim, hergün dualarında beni ilk siraya koyduğu büyük biliyorum! Beni her zor durumdan çıkarılan güzel duaların için çok teşekkür...
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