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DEVELOPMENT OF DNA DIAGNOSTICS OF NEGLECTED TROPICAL DISEASES IN RESOURCE-LIMITED SETTINGS

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DEVELOPMENT OF DNA DIAGNOSTICS OF NEGLECTED TROPICAL DISEASES IN RESOURCE-LIMITED SETTINGS

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General introduction

Point-of-care (PoC) diagnostic tests decentralize diagnostics by bringing diagnostic tests out of the laboratory and closer to the patient. PoC tests that do not rely on specialized equipment or infrastructure will enable rapid and reliable diagnostics for infectious diseases in resource-limited settings, where they are urgently needed. In this introduction, we introduce the concepts that are relevant for this thesis concerning PoC diagnostic tests for Neglected Tropical Diseases in resource-limited settings, and the Clustered Regularly Interspaced Short Palindromic Repeat system and its CRISPR-associated protein that we use to develop a DNA-based PoC diagnostic scheme.

1

1.1 Motivation: Developing diagnostics for neglected tropical diseases

The 2030 Agenda for Sustainable Development provides a roadmap to improve the lives of everyone worldwide in the form of the United Nations Sustainable Development Goals (Figure 1.1)[1]. This thesis focuses on goal number 3.3, which aims to end epidemics of AIDs, tuberculosis and malaria, as well as epidemics of neglected tropical diseases (NTDs) and other infectious diseases (e.g. communicable diseases such as hepatitis)[2]. More specifically, this thesis focuses on these NTDs, and diagnostics for them.

Figure 1.1: 17 United Nations Sustainable Development Goals adopted in 2015, set to be achieved by 2030 (Image source[1]).

Infectious diseases, caused by bacteria, fungi, or viruses, remain prevalent around the world[3]. They are transmitted from person to person, through contaminated food and water, or through zoonotic means via insects and animals. Although treatment for many infectious diseases exists, a lack of effective diagnostic tests, among other factors, impedes the treatment and ultimate eradication of infectious diseases[4]. Current diagnostic tests for infectious diseases generally require resources such as infrastructure, electricity, and expensive equipment such as microscopes and trained users. These resources are often not available in resource-limited regions in the world[5], for example, in remote regions in Kenya and Uganda where infectious diseases continue to spread (Figure 1.2)[6,7]. The term resource-limited settings, as defined by the World Bank, refers to resource-constrained regions (human, environmental and economic) with limited infrastructure and/or basic services in a low- or middle-income country. Therefore, populations living in resource-limited settings lack access to preventive (vaccines), diagnostic and/or therapeutic (treatment) care[8,9].

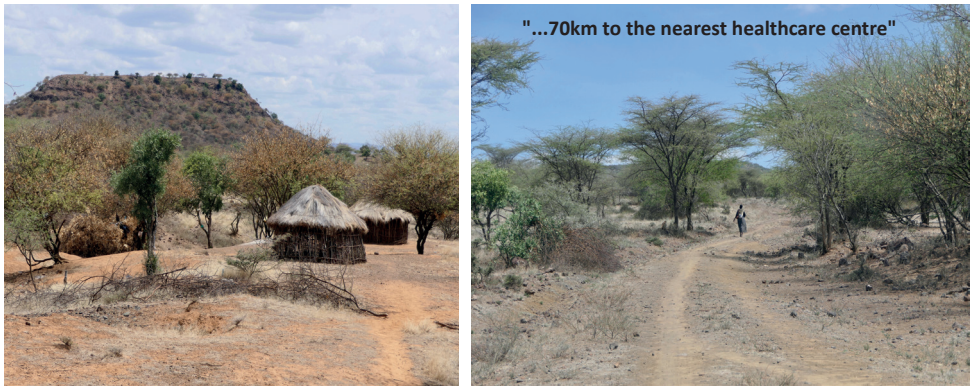


Figure 1.2: Remote resource-limited regions in East Pokot, Kenya. Photo credit: Astrid ten Bosch and Cees Dekker.

Indeed, multiple diseases remain endemic in resource-limited settings. NTDs are a diverse group of parasitic, viral, fungal, and bacterial infections, that are chronic, disabling, and potentially fatal[10]. They are prevalent in tropical and subtropical regions. Formally, there are 20 NTDs that are widely accepted in the scientific community, as selected by the World Health Organization (WHO) (Table 1.1)[11]. However, throughout the work presented in this thesis we refer to 24 individual NTDs as the leishmaniases (visceral and cutaneous); arbovirus infections (dengue and chikungunya); fungal infections (mycetoma and chromoblastomycoses), and human African trypanosomiasis (caused by two different species) are separated into different clinical forms or causative species due to their different diagnostic requirements – see the WHO NTD roadmap 2021-2030[11]. In general, NTDs are strongly associated with malnutrition, a weak immune system, and a lack of financial resources which perpetuate the spread of these NTDs[12].

Many countries and approximately 1 billion people (!) are affected by one or more NTDs[12] (Figure 1.3). Due to globalization, NTDs are no longer restricted to the poor and marginalized populations in resource-limited settings, but are increasingly affecting the populations in high-resource settings as well[10]. For example, visceral leishmaniasis has been reported in Italy and Spain[13]. Many NTDs still require effective diagnostic tests. Hence, there is a great need for simple, yet sensitive diagnostic tests, that are field deployable ("ready-to-use") and that can be easily implemented within a remote location. This will enable rapid and accurate diagnoses with minimal equipment and training, especially for infectious disease outbreaks that require rapid diagnostics[14].

Table 1.1: The WHO's list of 24 NTDs (based on their different diagnostic requirements). Daily adjusted life years (DALYs) is a measure to quantify the burden of a disease from mortality and morbidity[10]. One DALY is considered as one year of healthy life lost. The sum of DALYs across a population gives an indication of the difference between the current health status of that population and the ideal health status in the absence of the disease[4]. Information was adapted from [39].

Category	Disease	Approximate global prevalence (million)	DALYs lost
Helminths	Schistosomiasis	143 (2017)	2.5 million
	Soil transmitted helminthiases (*Ascaris*, hookworm,trichuriasis)	1.5 billion (2003)	3.5 million
	Dracunculiasis	<0.01 (2018)	ND
	Echinococcus	ND	871,000
	Taeniosis (cysticercosis)	5.5 (2010)	2.8 million
	Lymphatic filariasis	50 (2017)	1.2 million
	Onchocerciasis	21 (2017)	205 million
	Foodborne trematodiases	20-40 (2016)	2 million
Protozoa	Cutaneous leishmaniasis	<0.15 (2017)	260,000
	Visceral leishmaniasis	<0.03 (2017)	570
	Chagas disease	6-7 (2019)	219,000
	Human African trypanosomiasis (rhodesiense)	<0.01 (2017)	ND
	Human African trypanosomiasis (gambiense)	<0.01 (2017)	ND
Fungal	Mycetoma	ND	ND
	Chromablastomyosis	ND	ND
Bacterial	Buruli ulcer	<0.01 (2018)	ND
	Leprosy	0.2 (2017)	ND
	Yaws	<0.01 (2018)	ND
	Trachoma	142 (2019)	ND
Viral	Dengue	104 (2017)	3 million
	Chikungunya	2 (2004)	ND
	Rabies	<0.01 (2017)	1.6 million
Venom	Snakebite	2.7 (2015)	6-8 million
Ectoparasite	Scabies, other ectoparasites	455 (2016)	5.6 million

*ND denotes no data

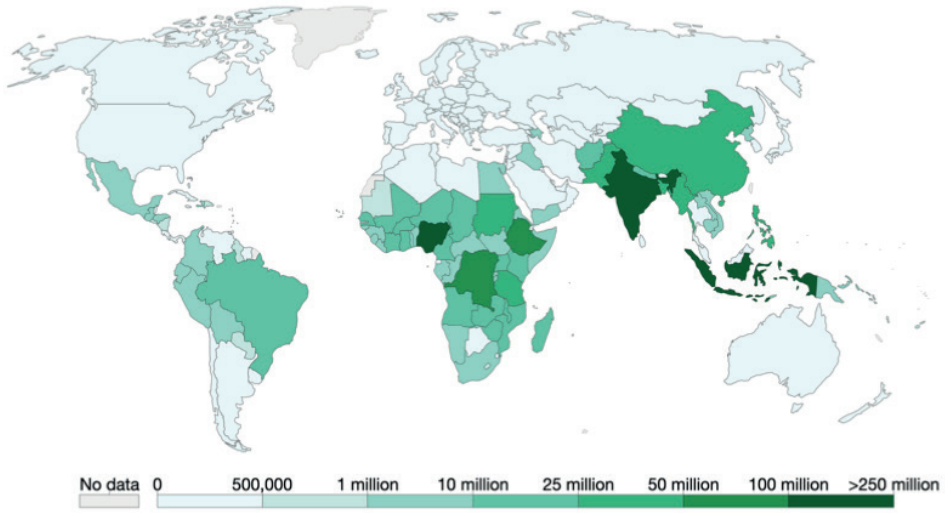


Figure 1.3: The number of people requiring interventions against NTDs (2015)[40].

This thesis focuses on diagnostics for visceral leishmaniasis (VL), an NTD which is caused by parasites from the *Leishmania* genus. *Leishmania* parasites are unicellular eukaryotes with a defined nucleus and organelles, such as a kinetoplast and flagella for motility[15] (Figure 1.4). Approximately 20 different *Leishmania* species are pathogenic to humans[16]. Leishmaniasis is transmitted to humans through the bite of infected female phlebotomine sandflies (main vector)[15], which are tiny insects (1.5-3.5mm in length) that are generally active at night. The lifecycle of *Leishmania* parasites occurs in the intestinal tract of sandflies. The parasite has two structural stages: the infective promastigote stage and the amastigote stage (Figure 1.5). A sandfly injects the infective promastigotes into a mammalian host, where they are phagocytized by macrophages. Promastigotes then transform into amastigotes in the macrophages, and subsequently multiply by simple division and continue to infect other phagocytic cells in the surrounding tissues of the host. Other sandflies then ingest amastigotes during blood meals which transform into promastigotes in the gut of the sandflies.

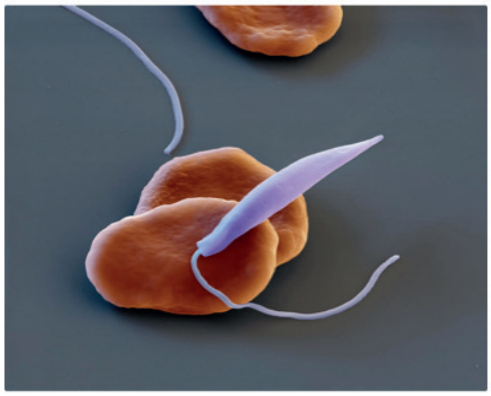


Figure 1.4: *Leishmania* parasite (purple) and red blood cells (red). Colour-enhanced scanning electron micrograph image (5,400X magnification)[41].

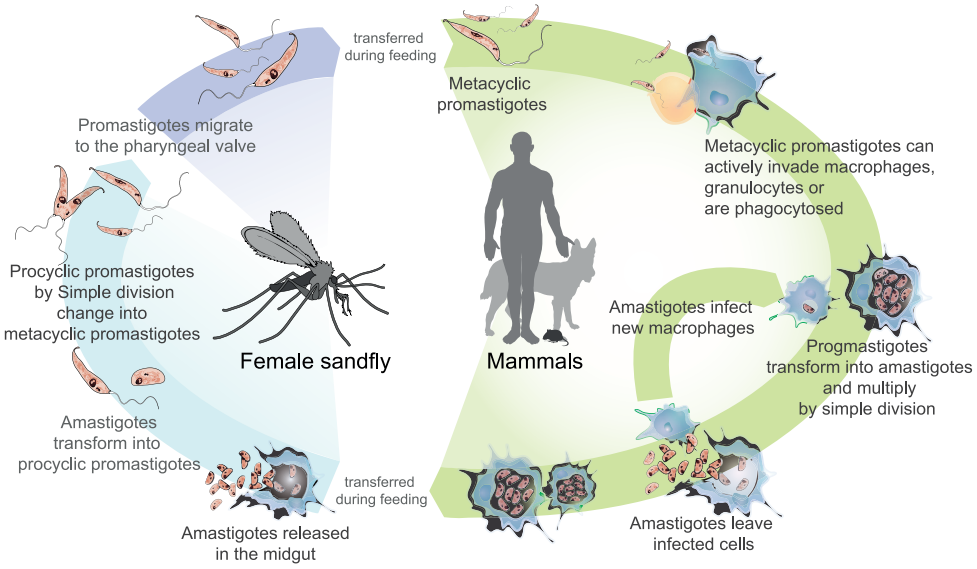


Figure 1.5: Schematic representation of the transmission cycle of the *Leishmania* parasite between sandflies and mammalian hosts[42].

Transmission of *Leishmania* parasites to humans via non-vector routes (e.g. blood transfusion, organ transplantation, or accidental laboratory infection) are possible but very rare. There are four clinical forms of this disease in humans; cutaneous, diffuse cutaneous, mucocutaneus, and visceral leishmaniasis which is also known as Kala-azar or black fever. VL is truly a disease of the poor as it affects the world's most marginalized populations. Given that VL is fatal if left untreated, effective diagnosis of this disease is of paramount importance.

1.2 Point-of-care diagnostic tests

Point-of-care (PoC) diagnostic tests solve this diagnostic problem by bringing diagnostics out of the laboratory and closer to the patients, therefore not relying on resources that are often not available in remote regions in the world where diagnostic tests are urgently needed (Figure 1.6)[9].

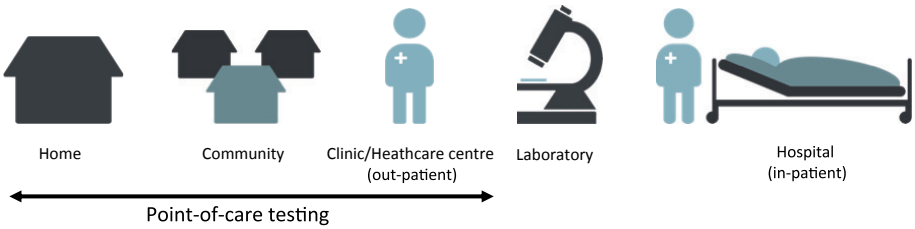
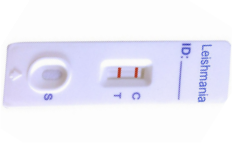


Figure 1.6: A schematic presentation of different diagnostic settings, indicating the ideal point-of-care diagnostic settings for resource-limited settings. Illustration credit: Mirte Vendel.

The introduction of affordable PoC tests, also known as rapid diagnostic tests (RDTs)[17], has had a major impact on solving the complex issue of accurately diagnosing febrile diseases[17]. Febrile diseases are difficult to diagnose, treat, and manage, because fever is one of the most common symptoms of many infections[18]. For infections that require treatment that is simple to administer (e.g. antimalarial medication), PoC tests are great screening tests. However, infections such as VL, require more reliable confirmatory tests, because the treatment itself is very toxic and requires intravenous administration and hospitalization[19]. Many febrile-presenting patients in resource-limited settings receive a cocktail of drugs on an empirical basis (based on symptoms)[17], including antibiotics. Reliable PoC tests would circumvent the unnecessary use of antibiotics which is especially important as we are in the era of increasing antibiotic resistance[18]. The use of PoC diagnostic tests will improve patient outcomes by enabling early and informed decisions about treatment regimens, and will aid medical professionals during infectious disease outbreaks, especially during critical times[4] such as the very recent COVID-19 pandemic.

PoC diagnostic tests are developing worldwide at a rapid rate. However current tests for VL remain relatively expensive, and require training and/or equipment[20]. The rk39 antigen-based test has been shown to have a poor performance in East Africa compared to India[21,22]. Current PoC diagnostic tests, such as the tests described for VL (Table 1.2), are often antibody-based which are less reliable as antibody production differs from person to person, and between countries. Antibody-based tests cannot distinguish between previous and current infections due to persistent antibodies that remain in the body after treatment[20,5]. More specific and reliable diagnostic tests exist, such as polymerase chain reaction (PCR)-based tests that probe for DNA, but they are not suitable as PoC tests for resource-limited settings as they require microscopes, sterile environments, and other advanced tools that are simply not available in these settings[20]. There are ample opportunities for further research and development of more reliable PoC diagnostic tests.

Table 1.2: Currently available serological VL diagnostic tests. Photo credit: Astrid ten Bosch.

Test	Target	Read-out	
IT Leish[44]	Antibodies against rK39 antigens that are present on the test	Immunochromatography: a dipstick is added to the sample	
Direct agglutination test (DAT)[45]	Antibodies against *Leishmania* species in a blood sample	Agglutination (i.e. aggregation)	
Latex agglutination test[46]	Antigens in urine samples	Agglutination of latex beads covered in IgG antibodies	
Onsite *Leishmania* Antibody rapid test[38]	Antibodies against rK39 antigens that are present on the test	Lateral flow assay	

DNA-based diagnostic tests are more reliable and accurate as they directly detect the presence of the pathogen which is independent of the patient's immune response. However, there are a limited number of DNA-based PoC diagnostic tests that have been developed. One of the main limitations of PoC tests is the sensitivity of the tests. Isothermal amplification is a technique that enables enhanced sensitivity of PoC tests by amplifying the DNA that is present in the sample[23]. Isothermal amplification enables DNA amplification at a constant temperature, without the need for expensive equipment such as a thermal cycler (PCR machine)[24]. Although these techniques commonly use a heating block, isothermal amplification designs are continuously improving to avoid all equipment. Isothermal amplification techniques include recombinase polymerase amplification (RPA), strand displacement amplification (SDA), rolling circle amplification (RCA), multiple displacement amplification (MDA), loop-mediated isothermal amplification (LAMP), helicase-dependent amplification (HDA), and nucleic acid sequence-based amplification (NASBA)[25,26]. The main features of each method are summarised in table 1.3. RPA is compatible with lyophilization (freeze drying), which is a process in which water is removed from a product and placed in a vacuum, allowing the product to be stored in a functional state for extended periods of time[26]. Methods that are compatible with lyophilization are very attractive for PoC diagnostics as it extends the shelf-life of the PoC test.

Isothermally amplified DNA in a sample can then be subjected to DNA detection. To achieve DNA detection for PoC diagnostics, scientists have extensively repurposed naturally occurring biological systems to detect DNA in samples, such as the use of DNA-binding proteins to probe for the DNA of a pathogen (bacterium, virus or any other organism that causes a disease) in patient samples[27].

Table 1.3: Isothermal amplification methods[25,26]. ND denotes no data.

Method	Incubation temperature (°C)	Initial heating	Incubation time (min)	Limit of detection (copies)
RPA	25-45	No	20-40	1
SDA	30-55	No	60-120	10
RCA	30-65	No	60-240	10
MDA	30-40	Yes	12-16 HRS	ND
LAMP	60-65	Yes	60	5
HDA	65	No	30-120	1
NASBA	41	No	60-180	1

1.3 CRISPR-Cas9

For the technology underlying the PoC test that we develop in this thesis, we need to zoom in on DNA-protein interactions. Humans have an adaptive immune system that protects us from invading pathogenic organisms that cause diseases, such as bacteria, viruses and parasites. Similarly, bacteria have an adaptive immune system that is known as the Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR) and their CRISPR-associated (Cas) proteins. CRISPR-Cas systems protect bacteria from invading organisms, such as bacteriophages (viruses that attack bacteria), by inactivating the invading organisms[28]. This process occurs in three stages: adaptation, maturation, and interference. As the invading virus infects a bacterium, a small fragment of the viral DNA is removed and stored in a library known as the CRISPR array – which is essentially a memory bank of previous viral infections. This is the first stage of CRISPR immunity known as the adaptation stage which allows the bacterium to defend itself against the invading virus upon reinfection. In the second stage of immunity, known as maturing, these short fragments are transcribed into CRISPR RNAs where the bacterium produces a complementary piece of RNA, hereafter known as the guide-RNA (gRNA), which associates with the Cas protein to form a "search" complex. The Cas9 protein, in complex with the gRNA, can then act upon and cleave the foreign DNA in a manner that is analogous to a pair of scissors, which is the third and final stage of CRISPR adaptive immunity known as interference (Figure 1.7)[28,29].

There are two main classes of CRISPR systems, which are further divided into different types. Class 1 systems are characterized by large multi-subunit protein complexes that conduct the host defence. Class 2 systems are characterized by

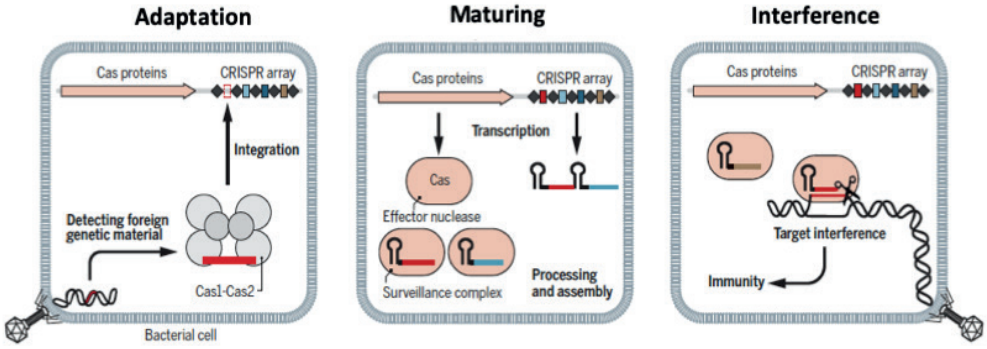


Figure 1.7: Schematic overview of CRISPR-Cas adaptive immunity. Adaptation is the process in which foreign viral fragments are incorporated into the CRISPR array. Maturing is the process in which the CRISPR array and Cas proteins are expressed to form the "search complex". Interference is the process in which the Cas protein in complex with the gRNA cleave and inactivate the foreign viral DNA. Image adapted from[47].

a single protein that conducts host defense[23]. CRISPR-Cas9 is a prominent class 2 system that is simple to program as it is a two-component system that can be reprogrammed simply by equipping the gRNA with different targeting sequence. Therefore, CRISPR-Cas9 has gained immense attention for applications such as genome editing and molecular diagnostics. The CRISPR-Cas9 complex binds to double stranded DNA (dsDNA) targets. Upon binding of the Cas9 protein to dsDNA, the complex initiates directional unwinding of the target DNA and subsequent base pairing with the gRNA. The Cas9 protein then cleaves both strands of the target DNA[23].

The most widely used Cas9 protein is from *Streptococcus pyogenes* (SpCas9), that is often complexed with a single chimeric guide RNA (sgRNA) (Figure 1.8)[30]. SpCas9 targets a dsDNA sequence via complementarity to a 20 nucleotide (nt) sequence that is flanked by a 3nt protospacer adjacent motif (PAM). ApoCas9 adopts a bilobed structure that comprises an alpha-helical recognition (REC) lobe and a nuclease (NUC) lobe that is connected by an arginine-rich bridge helix. The REC lob is divided into three domains (REC1, REC2, REC3). The NUC lobe contains the PAM-interacting (PI) domain, and two nuclease domains (HNH and RuvC) which function as molecular scissors to cleave the target and non-target strands, respectively, in the presence of the divalent cation magnesium (Mg^{2+})[30].

CRISPR-Cas systems are the basis of many recent DNA-based PoC diagnostic tests[31], such as Sherlock and DETECTR diagnostic tests (Table1.4)[32,33]. While CRISPR-Cas-based detection schemes are extremely promising diagnostic tools, these tools are yet to be validated in the field. There are very strict guidelines and regulatory processes for a diagnostic scheme to progress from proof-of-principal to a product that can be safely used in the field. It is imperative to ensure that a novel PoC test fits the local context in which the test will be used.

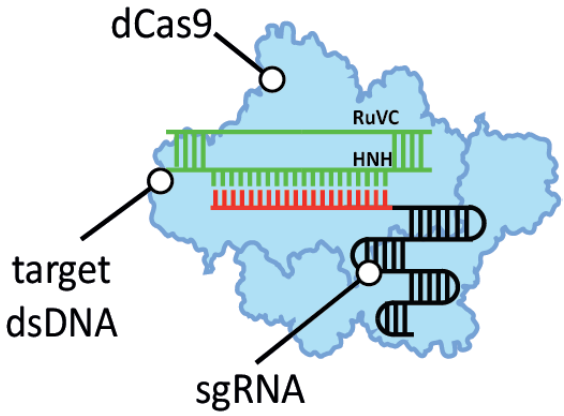


Figure 1.8: Schematic of Cas9 and sgRNA complex bound to a dsDNA target.

Table 1.4: Summary of CRISPR-Cas-based detection schemes with selected features.

CRISPR-Cas Detection	Name of detection scheme	Isothermal amplification	Additional technique	Readout	Target	Time
Cas9 [48]	-	NASBA	Toehold switch	Colorimetric – naked eye	Zika and Dengue	3H
Cas9 [49]	Paired Cas reporter	-	PCR	Bioluminescence	Tuberculosis	~1H
Cas9 [50]	CRISPR-Cas9 triggered isothermal exponential amplification reaction	EXPAR	-	Fluorescence	DNA methylation	<1H
Cas9 [51]	RCA-CRISPR-Split-HRP	RCA	-	Colorimetric – naked eye	miRNA (cancer marker)	<4H
Cas9 [52]	CRISPR-Cas9 triggered nicking endonuclease-mediated strand displacement amplification	SDA	Peptide nucleic acid	Fluorescence	SNP	>3H
Cas13a/ C2c2 [53]	Specific High Sensitivity Enzymatic Reporter UnLOCKing	(RT) RPA	T7 transcription (DNA to RNA)	Fluorescence	Zika and Dengue	~5H
Cas13a/ C2c2 [54]		(RT) RPA	Csm6 cleavage	LFA	Zika and Dengue	~3H
Cas13a[55]	Heating Unextracted Diagnostic Samples to Obliterate Nucleases	(RT) RPA	Combined with Sherlock detection	Fluorescence or LFA	Dengue and HIV	2H
Cas12a[56]	DNA endonuclease-targeted CRISPR trans reporter	RPA	-	Fluorescence	HPV	1H
Cas12a[57]	one-Hour Low-cost Multipurpose Highly Efficient System	-	PCR	Fluorescence	SNP	1H
Cas12b[58]		LAMP	-	Fluorescence	SNP	1H

Exponential amplification reaction (EXPAR); Reverse transcription (RT); lateral flow assay (LFA); Human Papillomavirus (HPV); single nucleotide polymorphism (SNP); hour (H); horseradish peroxidase (HRP).

1.4 Context-driven design for the end-user

There are multiple guidelines that are available to guide researchers through the development of diagnostic tests. The WHO's Sexually Transmitted Diseases Diagnostics Initiative (SDI) developed the ASSURED criteria as a benchmark for diagnosis, which stands for affordable (by those at risk of infection), sensitive (few false-positives), specific (few false-negatives), user-friendly (requiring minimal training), rapid (to enable treatment at the first visit) and robust (does not require refrigerated storage), equipment-free (compact and battery operated), and deliverable to end-users (especially to those who need it)[34]. In addition to the ASSURED criteria, target product profiles (TPPs) are also guidelines that are formulated by experts, such as the WHO and organizations such as the Foundation for Innovative New Diagnostics (FIND)[18,35]. TPPs are very specific guidelines that outline the minimal and optimal features of a diagnostic test. Essentially, PoC diagnostic tests need to be designed for the end-user, and the needs of the end-user will differ depending on the context in which the PoC diagnostic test will be used[18]. For example, a PoC diagnostic test that is designed for use within remote tropical regions, such as Kenya and Uganda, will need to have a broad temperature range of operation. However, a PoC diagnostic test that is designed for use within the Netherlands will not require such a broad temperature range as cold-chains (temperature-controlled refrigeration) are readily available[9], and tests are generally done near 20°C. Hence, the design specifications of a new PoC diagnostic test need to be adaptable to address the needs of the end-user[36,37]. Thus, we as researchers need to conduct direct observations during field research in remote resource-limited settings, to determine the design specifications for newly developed PoC tests.

1.5 Scope and objective of this dissertation

The aim of this thesis was to develop a DNA detection scheme for a PoC diagnostic test for NTDs for use within resource-limited settings. The scientific innovation is to develop an adaptable DNA-based detection scheme, using CRISPR-dCas9 (catalytically inactive Cas9), that can detect the DNA of any pathogen in bodily fluids i.e., in a blood or urine sample. This detection of DNA of the pathogen will be much more reliable than antibody-based tests as it will work independently of the persons immune response. Unlike current antibody-based diagnostic tests, it will be able to distinguish between current and previous infections. Specifically for VL, the current rk39 antigen-based RDT lacks specificity and sensitivity in sub-Saharan Africa, where VL remains prevalent[38]. We aim for a DNA-based detection scheme that does not require infrastructure, electricity, or skilled laboratory personnel to operate. Furthermore, the DNA-detection scheme will need to be functional at a broad temperature range, yet remain highly sensitive and specific. Such a DNA-detection scheme can be a promising tool for effective diagnosis of NTDs within

resource-limited settings, though it needs to be further tested, incorporated into a packaged test format, and validated in the field. Integrating this DNA-detection scheme into a potentially low-cost diagnostic test is a very promising alternative to current diagnostic tests in both high-resource and resource-limited settings.

The work presented in this dissertation is an original DNA-detection scheme that is coupled to a readout that is visible to the naked eye, thus enabling equipment-free PoC diagnoses. Next to describing the technical advances, this thesis presents an original observation of the healthcare systems in rural Kenya and Uganda, where PoC diagnostic tests are urgently needed, which provides design specifications for the DNA-detection scheme that we have developed. It is interdisciplinary as it included a collaboration with the Industrial Design Faculty to determine the design specifications of a new PoC test that cannot be found in literature.

In chapter 2 we present a review on the status quo of diagnostic tests and practices for all 24 NTDs addressed in the WHO's 2021-2030 roadmap, based on their different diagnostic requirements. We explore the capabilities and shortcomings of current diagnostic tests, identify diagnostic needs, and formulate prerequisites of relevant PoC tests. Next to technical requirements, we discuss the importance of availability and awareness programs for establishing PoC tests that fit endemic-resource limited settings.

In chapter 3 we present a field research study. We explore the local healthcare systems in rural resource-limited settings in Kenya and Uganda in order to understand how visceral leishmaniasis is diagnosed in these settings. More specifically, we explore the availability of resources (i.e., infrastructure, electricity, skills) in order to elucidate design specifications for a DNA-based PoC test. In this chapter, we present our stepwise approach which integrates elements of design thinking and that uses a combination of literature reviews and field research, which collectively present a context analysis of the healthcare systems and diagnostic practices in rural Kenya and Uganda. We explore two examples of visual thinking, namely Gigamaps and patient journeys, to communicate our findings from the field research with key stakeholders.

In chapter 4 we present the scientific methodology used to develop a DNA-based detection scheme. We explore the use of the CRISPR-dCas9 system as a DNA detection tool for diagnostics. For proof-of-principle, we selected the NTD visceral leishmaniasis as it is a fatal NTD that urgently requires improved PoC diagnostic tests in both rural Kenya and Uganda. Upon further development, this detection scheme could serve as a direct DNA-based PoC test for use within resource-limited settings such as described in chapter 3.

In chapter 5 we present a study that explores whether small RNA molecules can inhibit the catalytic activity of CRISPR-Cas9 in vitro. In chapter 4, we used CRISPR-dCas9 as a DNA-detection tool in our diagnostic platform, hence inhibiting its activity is of great interest. In this chapter we explore aspects of the CRISPR-Cas9 system that may have implications for genome engineering. Although this chapter does not directly relate to the development of the DNA-based detection scheme presented in chapter 4, we nonetheless explore the inhibition of CRISPR-Cas9, as the results can contribute to the overall knowledge of CRISPR-Cas applications.

In chapter 6, finally, I finish with a brief discussion of the adaptable potential of our DNA-detection scheme, and a general perspective.

References

1. Sustainable Development Goals .:. Sustainable Development Knowledge Platform. Available at: https://sustainabledevelopment.un.org (Accessed: 2nd April 2020).
2. WHO | SDG 3: Ensure healthy lives and promote wellbeing for all at all ages. WHO (2017).
3. Weerakoon, K. G. & McManus, D. P. Cell-Free DNA as a Diagnostic Tool for Human Parasitic Infections. Trends Parasitol. 32, 378–391 (2016).
4. Hotez, P. J., Pecoul, B., Rijal, S., Boehme, C. & Aksoy, S. Eliminating the Neglected Tropical Diseases : Translational Science and New Technologies. 1–14 (2016). doi:10.1371/journal.pntd.0003895.
5. Bezerra, G. S. N., Barbosa, W. L., Silva, E. D. da, Leal, N. C. & Medeiros, Z. M. de. Urine as a promising sample for Leishmania DNA extraction in the diagnosis of visceral leishmaniasis – a review. Brazilian J. Infect. Dis. 23, 111–120 (2019).
6. Ngure, P. K., Kimutai, A., Ng'ang'a, Z. W., Rukunga, G. & Tonui, W. K. A review of Leishmaniasis in Eastern Africa. J. Nanjing Med. Univ. 23, 79–86 (2009).
7. Olobo-Okao, J. & Sagaki, P. Leishmaniasis in Uganda: Historical account and a review of the literature. Pan Afr. Med. J. 18, 1–7 (2014).
8. Aranda-jan, C. B., Jagtap, S. & Moultrie, J. Towards A Framework for Holistic Contextual Design for Low-Resource Settings. 10, 43–63 (2016).
9. McNerney, R. Diagnostics for Developing Countries. Diagnostics (Basel, Switzerland) 5, 200–209 (2015).
10. Mitra, A. K. & Mawson, A. R. Neglected tropical diseases: Epidemiology and global burden. Trop. Med. Infect. Dis. 2, (2017).
11. World Health Organization. Ending the neglect to attain the Sustainable Development Goals–A road map for neglected tropical diseases 2021-2030. *Geneva World Heal. Organ.* (2020).
12. Picado, A. et al. Access to prompt diagnosis: The missing link in preventing mental health disorders associated with neglected tropical diseases. *PLoS Negl. Trop. Dis.* 13, e0007679–e0007679 (2019).
13. Hotez, P.J. Southern Europe's Plagues: Vector-Borne Neglected Tropical Diseases. *PLoS Negl. Trop. Dis.* 10, e0004243 (2016).
14. Peeling, R. W. & Mabey, D. Point-of-care tests for diagnosing infections in the developing world. (2010).
15. Akhoundi, M. et al. A Historical Overview of the Classification, Evolution, and Dispersion of Leishmania Parasites and Sandflies. PLoS Negl. Trop. Dis. 10, e0004349 (2016).
16. Maroli, M., Feliciangeli, M. D., Bichaud, L., Charrel, R. N. & Gradoni, L. Phlebotomine sandflies and the spreading of leishmaniases and other diseases of public health concern. Medical and Veterinary Entomology 27, 123–147 (2013).
17. El-Safi, S., Chappuis, F. & Boelaert, M. The Challenges of Conducting Clinical Research on Neglected Tropical Diseases in Remote Endemic Areas in Sudan. PLoS Negl. Trop. Dis. 10, e0004736–e0004736 (2016).
18. Dittrich, S. et al. Target Product Profile for a Diagnostic Assay to Differentiate between Bacterial and Non-Bacterial Infections and Reduce Antimicrobial Overuse in Resource-Limited Settings: An Expert Consensus. PLoS One 11, e0161721 (2016).
19. Schallig, B. Y. M. N. and H. D. F. H. Evaluation of Urine as a Diagnostic Specimen for

Visceral Leishmaniasis in Sudan. Journal of Tropical Diseases 1, 1–3 (2013).
20. Abeijon, C. et al. Development of a multiplexed assay for the detection of Leishmania donovani/Leishmania infantum protein biomarkers in the urine of patients with visceral leishmaniasis. J. Clin. Microbiol. (2019). doi:10.1128/JCM.02076-18.
21. Burza, S., Croft, S. L. & Boelaert, M. *Leishmaniasis. Lancet* 392, 951–970 (2018).
22. Chappuis, F. et al. Diagnostic Accuracy of Two rK39 Antigen-Based Dipsticks and the Formol Gel Test for Rapid Diagnosis of Visceral Leishmaniasis in Northeastern Uganda Diagnostic Accuracy of Two rK39 Antigen-Based Dipsticks and the Formol Gel Test for Rapid Diagnosis of *Vis. J. Clin. Microbiol*. 43, 5973–5977 (2005).
23. Sashital, D. G. Pathogen detection in the CRISPR–Cas era. Genome Med. 10, 32 (2018).
24. Rosser, A., Rollinson, D., Forrest, M. & Webster, B. L. Isothermal Recombinase Polymerase amplification (RPA) of Schistosoma haematobium DNA and oligochromatographic lateral flow detection. 1–5 (2015). doi:10.1186/s13071-015-1055-3.
25. Deng, H. & Gao, Z. Bioanalytical applications of isothermal nucleic acid amplification techniques. Anal. Chim. Acta 853, 30–45 (2015).
26. Zanoli, L. M. & Spoto, G. Isothermal amplification methods for the detection of nucleic acids in microfluidic devices. Biosensors 3, 18–43 (2012).
27. Batista, A. C. & Pacheco, L. G. C. Detecting pathogens with Zinc-Finger, TALE and CRISPR- based programmable nucleic acid binding proteins. J. Microbiol. Methods 152, 98–104 (2018).
28. Strutt, S. C., Torrez, R. M., Kaya, E., Negrete, O. A. & Doudna, J. A. RNA-dependent RNA targeting by CRISPR-Cas9. Elife 7, 1–17 (2018).
29. Anders, C. & Jinek, M. In Vitro Enzymology of Cas9. The Use of CRISPR/cas9, ZFNs, TALENs in Generating Site Specific Genome Alterations 546, (Elsevier Inc.).
30. Zuo, Z. & Liu, J. Allosteric regulation of CRISPR-Cas9 for DNA-targeting and cleavage. Curr. Opin. Struct. Biol. 62, 166–174 (2020).
31. Chiu, C. In Translation Cutting-Edge Infectious Disease Diagnostics with CRISPR. 2015–2017 (2018).
32. Zhou, L., Peng, R., Zhang, R. & Li, J. The applications of CRISPR / Cas system in molecular detection. 5807–5815 (2018). doi:10.1111/jcmm.13925.
33. Li, Y., Li, S., Wang, J. & Liu, G. CRISPR/Cas Systems towards Next-Generation Biosensing. Trends Biotechnol. 37, 730–743 (2019).
34. Peeling, R. W., Holmes, K. K., Mabey, D. & Ronald, A. Rapid tests for sexually transmitted infections (STIs): the way forward. *Sex. Transm. Infect*. 82, v1 LP-v6 (2006).
35. Cruz, I. et al. Target Product Profile for a point-of-care diagnostic test for dermal leishmaniases. Parasite Epidemiol. Control 5, e00103 (2019).
36. Cruz, I. et al. Target Product Pro fi le for a point-of-care diagnostic test for dermal leishmaniases. 3, 1–6 (2019).
37. Engel, N. et al. Addressing the challenges of diagnostics demand and supply: insights from an online global health discussion platform. BMJ Glob. Heal. 1, e000132 (2016).
38. Bezuneh, A. et al. Comparison of point-of-care tests for the rapid diagnosis of visceral leishmaniasis in East African patients. *Am. J. Trop. Med. Hyg.* 91, 1109–1115 (2014).
39. WHO disease packs 2030. Available at: https://www.who.int/neglected_diseases/news/NTD_disease_packs.pdf?ua=1. (Accessed: 11th June 2020).
40. Number of people requiring interventions against neglected tropical diseases (NTDs), 2015. Available at: https://ourworldindata.org/grapher/interventions-ntds-sdgs. (Ac-

cessed: 2nd April 2020).
41. Leishmaniasis And Red Blood Cell, Sem Photograph by Eye of Science. Available at: https://fineartamerica.com/featured/2-leishmaniasis-and-red-blood-cell-sem-eye-of-science.html. (Accessed: 16th April 2020).
42. File:Leishmaniasis life cycle diagram en.svg - Wikimedia Commons. Available at: https://commons.wikimedia.org/wiki/File:Leishmaniasis_life_cycle_diagram_en.svg. (Accessed: 21st August 2020).
43. Mabey, D., Peeling, R. W., Ustianowski, A. & Perkins, M. D. Diagnostics for the developing world. Nat. Rev. Microbiol. 2, 231–240 (2004).
44. Peruhype-Magalhães, V., Machado-de-Assis, T. S. & Rabello, A. Use of the Kala-Azar Detect® and IT-LEISH® rapid tests for the diagnosis of visceral leishmaniasis in Brazil . Memórias do Instituto Oswaldo Cruz 107, 951–952 (2012).
45. Adams, E. R. et al. Leishmaniasis Direct Agglutination Test: Using Pictorials as Training Materials to Reduce Inter-Reader Variability and Improve Accuracy. PLoS Negl. Trop. Dis. 6, e1946 (2012).
46. Attar, Z. J. et al. Latex agglutination test for the detection of urinary antigens in visceral leishmaniasis. Acta Trop. 78, 11–16 (2001).
47. Knott, G. J. & Doudna, J. A. CRISPR-Cas guides the future of genetic engineering. Science (80-.). 361, 866 LP – 869 (2018).
48. Pardee, K. et al. Rapid, Low-Cost Detection of Zika Virus Using Programmable Biomolecular Components In Brief Rapid, Low-Cost Detection of Zika Virus Using Programmable Biomolecular Components. Cell 165, 1255–1266 (2016).
49. Zhang, Y. et al. Paired Design of dCas9 as a Systematic Platform for the Detection of Featured Nucleic Acid Sequences in Pathogenic Strains. ACS Synth. Biol. 6, 211–216 (2017).
50. Huang, M., Zhou, X., Wang, H. & Xing, D. Clustered Regularly Interspaced Short Palindromic Repeats/Cas9 Triggered Isothermal Amplification for Site-Specific Nucleic Acid Detection. Anal. Chem. 90, 2193–2200 (2018).
51. Qiu, X. et al. Highly E ff ective and Low-Cost MicroRNA Detection with CRISPR-Cas9. (2018). doi:10.1021/acssynbio.7b00446.
52. Zhou, W. et al. A CRISPR–Cas9-triggered strand displacement amplification method for ultrasensitive DNA detection. Nat. Commun. 9, 5012 (2018).
53. Gootenberg, J. S. et al. Nucleic acid detection with CRISPR-Cas13a / C2c2. 9321, (2017).
54. Gootenberg, J. S. et al. Multiplexed and portable nucleic acid detection platform with Cas13, Cas12a and Csm6. Science (80-.). 360, 439–444 (2018).
55. Myhrvold, C. et al. Field-deployable viral diagnostics using CRISPR-Cas13. Science (80-.). 360, 444–448 (2018).
56. Chen, J. S. et al. CRISPR-Cas12a target binding unleashes indiscriminate single-stranded DNase activity. Science (80-.). 360, 436–439 (2018).
57. Li, S.-Y. et al. CRISPR-Cas12a-assisted nucleic acid detection. Cell Discov. 4, 20 (2018).
58. Li, L. et al. HOLMESv2: A CRISPR-Cas12b-Assisted Platform for Nucleic Acid Detection and DNA Methylation Quantitation. ACS Synth. Biol. 8, 2228–2237 (2019).

2

Diagnosing point-of-care diagnostics for neglected tropical diseases

Inadequate and non-integrated diagnostics are the Achilles' heel of global efforts to monitor, control, and eradicate neglected tropical diseases (NTDs). While treatment is often available, NTDs are endemic among marginalized populations, due to the unavailability or inadequacy of diagnostic tests which cause empirical misdiagnoses. The need of the hour is early diagnosis at the point-of-care (PoC) of NTD patients. Here, we review the status quo of PoC diagnostic tests and practices for all of the 24 NTDs identified in WHO's 2021-2030 roadmap, based on their different diagnostic requirements. We discuss the capabilities and shortcomings of current diagnostic tests, identify diagnostic needs, and formulate prerequisites of relevant PoC tests. Next to technical requirements, we stress the importance of availability and awareness programs for establishing PoC tests that fit endemic resource-limited settings. Better understanding of NTD diagnostics will pave the path for setting realistic goals for healthcare in areas with minimal resources, thereby alleviating the global healthcare burden.

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2.1 Introduction: Point-of-care diagnostics

The International Organization for Standardization defines point-of-care (PoC) diagnostic testing as 'testing that is performed near or at the site of a patient with the result leading to a possible change in the care of the patient'.[1] In practical implementations, a PoC test is a specific and sensitive assessment wherein a user needs to administer a minimum number of steps to obtain an easy-to-interpret, rapid (within a short turn-around time (TAT)), and robust result. PoC tests are used everywhere within the health chain where there is need for a fast diagnostic outcome that is independent from sophisticated, often time-consuming, labour-intense, and expensive laboratory procedures. Ideally, PoC tests should be designed to function equipment-free as portable units with stable reagents that function efficiently within a broad range of environmental settings. Because of these favorable characteristics, PoC diagnostics is also known as 'bedside testing', 'remote rapid testing', 'near-patient laboratory testing', 'ancillary testing', and 'decentralized testing'. [2–4]

The ease of use of PoC diagnostics clearly exhibits potential to serve as an early diagnostic tool in resource-limited settings. Especially with recent advances in PoC diagnostics based on 'lab-on-a-chip' technology, PoC tests can be applied for triage and confirmatory diagnostics.[5,6] This circumvents issues such as shortage of healthcare staff and under-equipped laboratories, thereby improving clinical interventions which is especially advantageous in resource-limited settings. PoC diagnostics, especially when multiplexed, can reduce treatment costs, support disease surveillance, and minimize the unnecessary use of anti-microbials, thereby preventing the emergence of resistant strains.[5] Simple, rapid, and robust PoC diagnostic tests thus are a preferred choice for the diagnosis of Neglected Tropical Diseases (NTDs).

NTDs are diseases of poverty that affect more than a billion people worldwide. These communicable diseases are endemic to regions that have limited access to healthcare and hence, despite available treatments, they can be fatal. These diseases are referred to as 'neglected' as they receive inadequate attention when compared to other diseases such as malaria, human immunodeficiency virus, and tuberculosis.[7] The World Health Organization (WHO) has formally identified 20 NTDs.[8] However to accomodate diagnostic test requirements, the list was expanded in the 2021-2030 roadmap to 24 NTDs for control and elimination.[8] Although tremendous progress has been made in combatting NTDs over the past decade, they still prevail in high incidence numbers, significantly contributing to numerous disability-adjusted life years (DALYs) of afflicted populations (Fig. 1). In effect, NTDs pose the biggest disease burden to developing tropical economies.[9–13] The WHO's Sexually Transmitted Diseases Diagnostics Initiative (SDI) developed the ASSURED criteria as a benchmark for diagnosis, which stands for affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free, and deliverable to end-users[14].

Recent advances in the PoC diagnostics highlighted the issues with data and sample collection, therefore, new term REASSURED was coined comprising of real-time connectivity) and ease of specimen collection and environmental friendliness to the existing ASSURED criteria[15]. Furthermore, disease-specific guidelines called 'target product profiles' have been developed for certain NTDs. Unfortunately, despite multiple guidelines such as these, there is a lack of commercially viable diagnostic tests for NTDs. [16–20] Field-deployable PoC tests would facilitate early disease diagnoses, which are crucial for NTDs control and treatment, and for designing strategies for the gradual elimination of these endemic diseases.

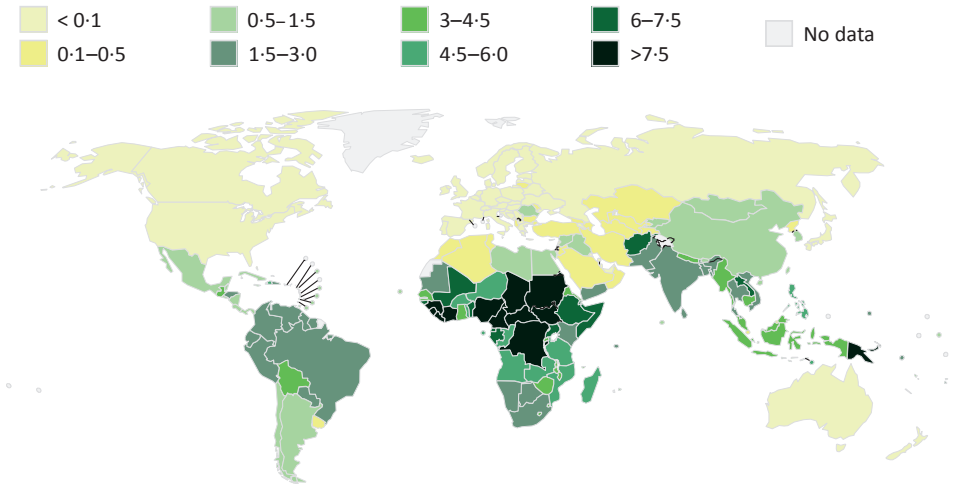

Figure 2.1: Spread of Neglected Tropical Diseases. Cumulative disability adjusted life years of afflicted populations due to the NTDs human african trypanosomiasis, chagas disease, schistosomiasis, leishmaniasis, lymphatic filariasis, onchocerciasis, taeniasis cysticercosis, echinococcosis, dengue, trachoma, rabies, leprosy, and soil-transmitted helminthiases. World map adapted from the WHO.[8]

In this review, we first discuss different technical approaches for PoC diagnostic tests, i.e., their basic working principle, advantages, and limitations. Next, in the core of this review, we outline the status quo of PoC NTD diagnostics, highlighting the implementation needs for various PoC NTD diagnostics. We end with a discussion and recommendations for future developments, to further accelerate the goal of achieving efficient NTD diagnostics.

2.2 Three different approaches for PoC diagnostics

At the heart, PoC diagnostics is an approach to identify, and possibly quantify a specific analyte. Based on the variety of biomolecular ways for detection of specific analytes, PoC tests can be classified into immunological tests, nucleic-acid-based

tests, and other (biomarker-based) tests (Fig 2) – which we describe below.

2.2.1 Immunological PoC tests

Immunological PoC tests detect the presence of an antigen (or its molecular counterpart) or an antibody that is generated as an immune response to an infection. In these PoC tests, an antibody/antigen immobilized on a solid substrate binds to the specific analyte, forming an immune-complex that subsequently generates a signal that is visible to a naked eye (Fig 2). The specific antigen-antibody interactions that are at the core of these immunosensors allow for both qualitative detection and quantitative assessment.

Immunological PoC tests have numerous advantages and hence are among the most commonly used tests. In addition to a quick TAT of ~15 minutes, these PoC tests do not require sample preparation and thus a clinical sample can be administered onto the test pad directly. Immunological PoC tests are field deployable as they are simple, rapid, easy-to-use, equipment-free, and robust, thus allowing administering at decentralized locations in an NTD-endemic region. Moreover, immunological PoC tests are cost effective compared to the other PoC approaches[21] discussed below.

Immunological PoC tests also have limitations.[22] Due to cross-reactivity, auto-antibodies and rheumatoid factors can reduce the efficacy of antigen-based PoC tests, as these molecules can specifically bind to free antigen in the sample and thereby block them from interacting with the specific antibody immobilized on the immunosensor. Antigen quantity also critically influences early disease diagnosis. While low antigen levels might result in false negatives, high levels of the antigen can cause 'prozoning' wherein excess antigen occupies most of the antibody-binding sites, thus resulting in false results. Antigen denaturation is another major factor affecting the lifetime and functionality of antigen-based PoC tests. Furthermore, antibody-based tests in general cannot be used as a confirmatory test or as a test-of-cure because they can provide false positive results due to the presence of antibodies that persist post-infection in the body. While a confirmatory test, by definition, verifies a diseased state, a test-of-cure is used to validate the efficacy of the administered treatment and to distinguish relapse of a disease and re-infections. Furthermore, the growth cycle of a pathogen can influence the immune response. While fast-growing pathogens could elicit an immune response within a few days of infection, slow-growing pathogens could take weeks before a detectable amount of antibody can appear in the bodily fluid, resulting in false negative results. In many cases, pathogens can evade the immune system and reside in the body for weeks to months before a detectable immune response is generated (asymptomatic carriers). Likewise, the overall efficiency of an immunological PoC test may be influenced by the varying immune responses of individuals within a population, especially when

afflicted populations are malnourished or have compromised immune systems due to co-morbidities. Finally, multiplexing immunological PoC tests is challenging as the test can favor one analyte over another, affecting the analytical efficiency. Immunological PoC tests are also not ideal to diagnose species specificity and to prescribe optimal treatment choices, for example in antibiotic therapy. Therefore, immunological PoC tests are mostly used as screening tests to select patients for subsequent confirmatory diagnosis.[2,23]

2.2.2 Nucleic-acid-based PoC tests (molecular PoC tests)

Nucleic-acid-based PoC tests probe for the presence of genetic material of the pathogen (DNA or RNA) to diagnose specific diseases. While specific amplification of the pathogens genetic material for disease diagnoses can be performed using nucleic-acid amplification tests (NAATs), mapping of the pathogen sequence for diagnosis is commonly done by next-generation sequencing (NGS) of the DNA/RNA (Fig 2). Commercially available PoC NAATs are based on the polymerase chain reaction (PCR) that occurs in a closed automated device which utilize pre-packaged single-use integrated cartridges filled with reagents for the nucleic-acid amplification.[2] The user merely needs to load the sample fluid and start the reaction by pressing a button. The entire PCR reaction then proceeds inside the device and the result is displayed within a TAT of 20-60 minutes on a screen. Such small, portable, and battery/power-operated PCR units can be used for the detection of a range of pathogens by simply adapting the cartridges.[24,25]

With the increasing demand for field-deployable PoC molecular diagnostic tests, new isothermal NAATs are being designed that, unlike PCR, do not require a thermocycler but instead function at a constant temperature, for example at 37°C or even room temperature.[26,27] Examples of commonly used isothermal NAATs are strand-displacement amplification, loop-mediated isothermal amplification (LAMP), and recombinase polymerase amplification (RPA), techniques that allow the amplification and detection within a TAT of 20 minutes. As these methods may suffer from relatively low specificity, novel CRISPR systems are being developed to function downstream of isothermal NAATs to enhance the specificity.[17] Capable of multiplexing, such detection systems could potentially become the best PoC molecular diagnostic toolbox. However, so far they have been restricted to laboratory diagnostics and further development is needed to adapt them into field-deployable PoC tests.[28–32] Portable NGS devices are one of the most promising candidates for efficient PoC diagnostics in the near future. Similar to NAATs, innovative NGS technologies have been developed for non-trained personnel with simplified handling, fast and accurate results, and reduced sample volumes.[33]

Nucleic-acid-based PoC tests provide a range of advantages. Given their very high

sensitivity and specificity, they can detect even a single pathogen in a biological sample. Furthermore, they lend themselves well for multiplexing. These PoC tests can serve as confirmatory diagnostics as they can identify the infection etiology, microbial resistance, and the extent of an infection (virulence) – thereby accelerating the choice of treatment.[34] Limitations of nucleic-acid-based PoC tests are mostly found in the pre-treatment of the samples. Owing to the extreme sensitivity of nucleic-acid-based tests, uncontaminated input samples need to be administered in a closed automated unit to avoid false positive results.[35] A further challenge can be to ensure accurate detection of traces of microbial nucleic-acids within the pool of other components in the biological sample, say the human genome, to avoid false negative results. A related challenge is to ensure specificity by identifying a unique target for the pathogen's DNA that does not exhibit any homology with other genomes in the sample. At this point in time, nucleic-acid-based PoC tests are too costly for resource-limited settings.[36] On top of that, additional costs due to hardware infrastructure for data handling may be a concern in such settings.[33]

2.2.3 Biomarker-based PoC tests

Biomarkers are biological molecules (other than antigens) that occur naturally in living beings and can be used to indicate a diseased state when deviations occur in their concentrations from the physiologically normal value. In this review, we refer to biomarkers as any molecule other than the antigen itself or the antibody that is elicited in response to the infection (cf. section 2.2.1) or the pathogen's nucleic-acid material (cf. section 2.2.2). While a plethora of biomarkers has been identified for infectious disease diagnosis,[37,38] C-reactive protein is so far the only biomarker that is available as a commercial PoC test for infectious diseases, and it is used for selecting effective anti-microbial treatment in acute respiratory-tract infections.[2]

New opportunities for biomarker-based PoC tests are emerging with aptamers – specific oligonucleotide sequences that bind a biomarker with high affinity. Since aptamers are synthetically produced, unlike antibodies, they are highly reproducible, cost-effective, and easy to modify. Aptamers are thermostable and can be reversibly denatured, making them a potent biosensor for PoC tests (Figure 2.2). These 'aptasors' have, for example, been developed for aptamer-linked immunosorbent assays or enzyme-linked oligonucleotide assays also known as enzyme-linked aptamer assays.[39-41]

The main advantages of biomarker-based PoC tests are that they can provide fast and accurate results.[37,41] Quite some research is on-going to develop biomarker-based PoC tests for a confirmatory diagnosis. A major disadvantage is the higher costs in the development and commercial applicability of biomarker-based PoC tests compared to immunological PoC tests.[2]

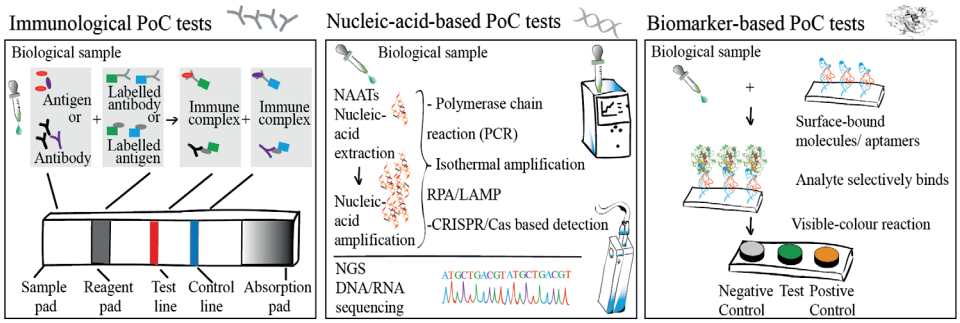

Figure 2.2: Three types of PoC diagnostic tests. A biological sample (such as blood, urine, saliva, sweat etc.) can be utilized for various types of PoC diagnosis. Left: Immunological PoC test. A biological sample is dropped onto the sample pad of a lateral flow assay, which acts as a filtering unit to sieve out unnecessary constituents. Upon administration of a reaction buffer (or its automated release), the analyte flows through the reagent pad, wherein an antigen antibody complex is formed. Driven by capillary action, this complex migrates to the next zone with control and test lines. While the appearance of a visible color at the test line confirms the infection, the control line signal ensures the test functionality. Middle: Nucleic-acid-based PoC test. Here, genetic material of a pathogen serves as the analyte. DNA/RNA from the pathogen is extracted from infected host cells, or circulating cell free within the clinical sample, especially urine. While extracted RNA is first reverse transcribed to obtain cDNA, extracted DNA can be directly amplified using PCR or using isothermal amplification (e.g., RPA or LAMP), typically in a fully automated portable unit (top-right). In some systems, the amplified DNA is then used for CRISPR/Cas recognition or other downstream processing to yield a diagnostic result within a lateral flow assay or a microfluidic lab-on-a-chip device. Next generation DNA/RNA sequencing (NGS) can also be utilized to identify specific diseases using a portable sequencer (bottom-right). Right: Biomarker-based PoC test. A biological sample is administered onto the test pad that in this case has specific surface-bound molecules such as aptamers that target the analyte. Upon successful interactions, a visible color read-out is obtained. The test can be in the form of separate wells ('lab in a well', bottom-left).

2.3 PoC tests for neglected tropical diseases

Owing to the advantages of PoC tests, they are highly preferred for diagnosing NTDs in resource-limited settings. NTD diagnostics is challenging since these diseases often strongly relate to the context, which includes the needs and capabilities of the end-users, available resources and infrastructure, extent of disease endemicity, and treatment options.[20] Successful implementation of NTD PoC diagnostics must therefore consider, in addition to the technical efficiency, the context-specific requirements.

In this review, we comprehensively summarize the available literature for each of the 24 NTDs that were identified in the 2021-2030 roadmap of the WHO[8], focusing on their current diagnostics, specifically on PoC tests if available, and the implementation needs for future PoC diagnostics. An extensive overview is provided in the Supplementary information (SI). A detailed evaluation of the status quo of PoC diagnostics for all 24 NTDs facilitated us to formulate prerequisites of a PoC test that

would fit the disease-specific context. Next to recognizing the required technical improvements, complementary needs were identified based on the treatment and follow-up routines for the NTDs. A summary of the resulting information on the current PoC tests and implementation needs for future PoC tests is presented in Table 1.

We found that 5 NTDs do not have any commercially available PoC tests, 16 NTDs are currently diagnosed with immunological PoC tests, 7 NTDs are currently diagnosed with nucleic-acid-based tests, and only 1 NTD (leprosy) is diagnosed with a proof-of-principle biomarker-based PoC test. The 5 NTDs for which no commercially available PoC diagnostic test is available are taeniasis, *rhodesiense* human african trypanosomiasis, dracunculiasis, and the skin NTDs mycetoma and chromoblastomycosis. For taeniasis, a new analyte for a PoC test must be identified that can serve as a biomarker for the presence of tapeworms and their larval forms, not only in humans but also in pork meat and water, to prevent ingestion of contaminated food and water. Likewise, for dracunculiasis, testing of water bodies for disease surveillance necessitates a field-deployable nucleic-acid-based test. For chromoblastomycosis, mycetoma and *rhodesiense* human african trypanosomiasis, novel PoC tests, ideally nucleic-acid-based, are required that can recognize causal species for efficient treatment. Although immunological-based PoC tests are available for the diagnosis of rabies, schistosomiasis, chagas disease, lymphatic filariasis, chikungunya, snakebite envenoming, dengue, trachoma and yaws, these are mostly found to be sub-optimal (see SI) and further development of more reliable tests for the diagnosis of these NTDs is needed. The most common need amongst these NTDs is found to be the field deployability, followed by confirmatory diagnosis and test-of-cure. Field deployability concerns the ability for a test to be used in the field within resource-limited settings (i.e. without the need for a laboratory). This includes characteristics such as reliability (stable storage at room temperature), reproducibility (low batch-to-batch variation), costs, and accessibility of the test. Both immunological and nucleic-acid-based PoC tests are available for foodborne trematodiases, echinococcosis, buruli ulcer, leishmaniasis (both visceral, and cutaneous), onchocerciasis, and *gambiense* human african trypanosomiasis. Interestingly, despite the availability of a PoC test, these tests need to be further optimized for use in the field, preferably for applications as simple as 'under a tree'. Dedicated field studies must be conducted to evaluate the performance of such PoC tests.

We grouped these 24 NTDs based on our observations on common technical requirements for developing novel PoC tests for efficient diagnosis, see Fig 3. The demand for confirmatory diagnosis and a test-of-cure was one of the most common implementation needs, as can be deduced from the requirement for robust PoC tests utilizing nucleic-acid-based detection (red ellipse), or recognizing novel biomarkers (green ellipse). Along with field validation of existing PoC tests (pur-

ple), robust confirmatory tests with capabilities to identify species specificity is a pre-requisite for efficient and effective diagnosis of visceral leishmaniasis and *gambiense* human african trypanosomiasis, especially because treatment regimens for this trypanosomiasis is species dependent. Likewise, new sensitive and specific tests such as nucleic-acid-based/biomarker PoC tests are required for the diagnosis of schistosomiasis particularly in case of low parasitemia. Nucleic-acid-based PoC tests would also be the ideal solution for diagnosing congenital and asymptomatic chagas disease. For taeniasis and *rhodesiense* human african trypanosomiasis, PoC tests with novel biomarker detection are required since there are currently no PoC tests available for these NTDs. For lymphatic filariasis, dengue and chikungunya, existing PoC tests are inefficient as they are cross-reactive with other infectious diseases (Table 1). Febrile illnesses dengue and chikungunya are often misdiagnosed as they occur as a seasonal outbreak within Zika endemic regions and hence novel PoC tests should be capable of multiplexing to distinguish between these viral illnesses, and thereby accelerate the diagnoses and minimize treatment delays. Similarly, onchocerciasis diagnosis would benefit from a confirmatory test and a test-of-cure. For the skin NTDs cutaneous leishmaniasis and buruli ulcer, sample preparation is a major concern as skin samples must be obtained in a sterile environment. Thus, novel PoC tests that could function with (non-invasive) surface scrapings of the skin tissue are required for efficient diagnosis here.

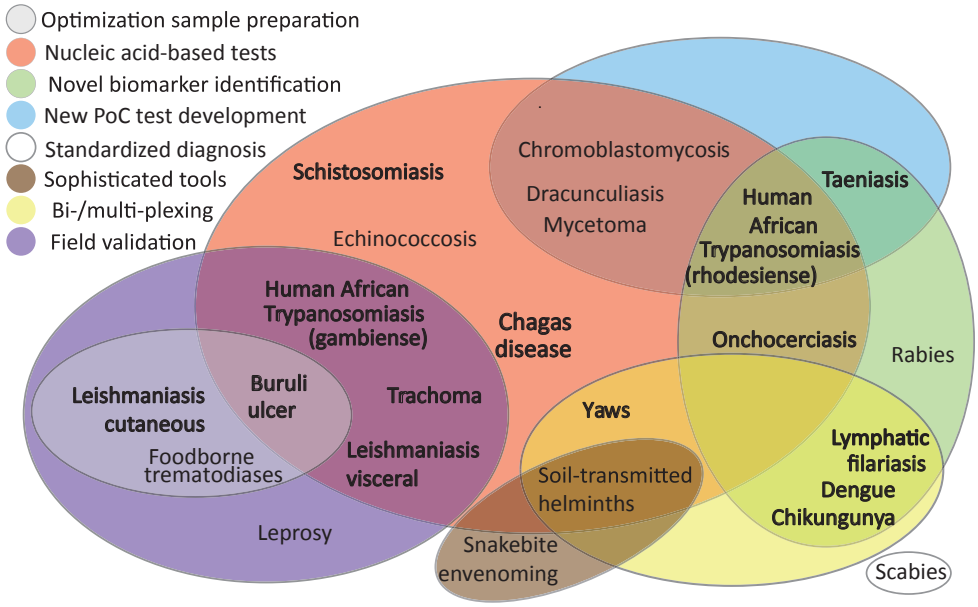

Figure 2.3: Venn diagram depicting the PoC implementation needs for various NTDs.
NTDs were placed in the colored circles based on their PoC implementation need. The legends indicate the particular diagnostic need of the NTD. NTDs in bold depict priority for PoC diagnoses as set in the WHO's 2021 2030 roadmap[8].

Table 2.1: NTD specific status quo of existing PoC test and future implementation needs for PoC tests. For each NTD, an overview is given of current PoC tests and the most urgent implementation needs. Current PoC tests are either listed according to their commercial product names or as proof-of-principle tests. NTDs that have several commercial PoC tests, such as echinococcosis, have been described in detail in the SI.

Disease Name	Current PoC tests	Implementation Needs	References
Foodborne trematodiases	◦2 antigen-based tests and 1 nucleic-acid-based test for *Fasciola gigantica* ◦3 nucleic-acid-based tests for *Opisthorchis viverrini* ◦1 antigen-based test for *Paragonimus spp* ◦1 antigen-based and 1 nucleic-acid-based test for Clonorchis sinensis	◦Effective field-deployable sample preparation as DNA extraction is limited to a laboratory ◦Quality assessment and field validation for all the current PoC tests	50–52
Taeniasis cysticercosis	◦ No commercial tests reported ◦ 1 antigen-based lateral flow test	◦Quality assessment and field validation for the proof-of-principle test ◦Novel biomarker identification ◦Field-deployable PoC test	53,54
Echinococcosis	◦Several commercial antibody tests for cystic and alveolar echinococcosis (e.g. (e.g. VIRAPID®HYDRATIDOSIS, ADAMUCE, RIDASCREEN®Echinococcus IgG test) ◦1 nucleic-acid-based LAMP test for cystic echinococcosis	◦More reliable PoC tests as current tests have low sensitivity and cannot detect inactive cysts ◦Confirmatory diagnostic tests for humans (test-of-cure) ◦Field-deployable screening tests for dogs	50,55
Rabies	◦Several commercial immunological tests (e.g. Vet-o-test Rabies Ag and Antigen Rapid Rabies Ag test kit	◦Novel circulating biomarker identification for dogs and humans to mitigate invasive sampling (brain tissue) ◦Field-deployable PoC test for humans ◦Quality assessment and field validation for all the current PoC tests	56-58
Chromoblas-to-mycosis	◦ No commercial tests reported	◦Field-deployable nucleic-acid-based test as species specificity will aid effective treatment	59
Leishmaniasis (cutaneous)	◦CL Detect™ Rapid Test (antibody-based) ◦Loopamp™ *Leishmania* detection it (nucleic-acid-based) ◦palmPCR (handheld battery-operated device) (nucleic-acid-based)	◦Field-deployable sample preparation is required for the Loopamp™ *Leishmania* Detection Kit and palmPCR ◦Quality assessment and field validation for all the current PoC tests	60-63
Mycetoma	◦ No commercial tests reported	◦Field-deployable nucleic-acid-based test as species specificity will aid effective treatment (bacterial treatment is more effective than fungal treatment)	64
Human African trypanoso-miases (*rhode-siense*)	◦ No commercial tests reported	◦Novel circulating biomarker identification ◦Field-deployable nucleic-acid-based test as species specificity will aid effective treatment	65-67

Buruli ulcer	◦Pilot immunological tests and molecular tests (LAMP and RPA)	◦Quality assessment and field validation ◦Field-deployable sample preparation ◦Confirmatory test (test-of-cure) is critical in cases of co-infections	68-70
Schistosomiasis	◦ Schisto POC-CCA® for *Schistosoma japonicum and Schistosoma mansoni* ◦*Schistosoma* ICT IgG-IgM rapid test ◦UCP-LA CAA assay	◦Field-deployable nucleic-acid-based test to enable the detection of low parasitaemia ◦Confirmatory test (test-of-cure) ◦Field-deployable antien-based test to detect *Schistosoma haematobium* ◦Ultra-sensitive field-deployable antigen-based test to detect low intensity *Schistosoma*	71-74
Chagas disease	◦Several commercial immunological tests (e.g. Chagas STAT-PAK assay, Chagas Detect™ *Plus* Rapid test (antigen-based), Trypanasoma Detect™)	◦Field-deployable nucleic-acid-based test to detect congenital chagas disease	13,75,76
Leishmaniasis (visceral)	◦IT-LEISH® Kit ◦Kalazar Detect™ ◦*Leishmania AB* Rapid test ◦VL-LFD device	◦A robust test for use in East Africa, where the IT-LEISH® Kit is not effective ◦Quality assessment and field validation of the mobile suitcase and VL-LFD ◦Field-deployable nucleic-acid-based test (test-of-cure), which is crucial for the diagnosis of post-kala-azar (PKDL)	77-79
Lymphatic filariasis	◦Alere™ Filariasis Test Strip (antibody-based) ◦SD BIOLOINE Lymphatic Filariasis IgG4 (antigen-based) ◦Brugia Rapid™ (antigen-based)	◦Field-deployable PoC test without cross reactivity with *Loa loa* ◦Multiplexed test with *Loa Loa* ◦Novel circulating biomarker identification	80,81
Chikungunya	◦SD BIOLINE Chikungunya (antigen-based) ◦Chikungunya IgM Combo Rapid test CE (antigen-based)	◦quality assessment and field validation ◦Field-deployable PoC test without cross reactivity with dengue ◦Multiplexed test with dengue ◦Novel circulating biomarker identification	82,83
Scabies	◦Burrow ink test ◦Handheld dermatoscopy test	◦A standardized diagnostic procedure will prevent misdiagnoses and delayed treatment	84-86
Onchocerciasis	◦SD BIOLINE *Onchocerciasis* IgG4 (antigen-based) ◦SD BIOLINE *Onchocerciasis* / LF biplex test with *Onchocerca volvulus* and *Wuchereria bancrofti* (antigen-based)	◦Field-deployable PoC test without cross reactivity with *Loa Loa* as this co-infection affects the treatment regimen ◦Novel circulating biomarker identification ◦Confirmatory test (test-of-cure) to aid surveillance in the current eradication era	87
Human African trypanosomiases (*gambiense*)	◦SD BIOLINE HAT and SD BIOLINE HAT 2.0 (antigen-based) ◦HAT Sero *K*-Set (antigen-based)	◦Quality assessment and field validation ◦Field-deployable nucleic-acid-based test as species specificity will aid effective treatment ◦Confirmatory test (test-of-cure)	88,89
Snakebite envenoming	◦1 test for 5 Australian species (antibody-based)	◦PoC test that is multiplexed for species that are geographically distinct ◦A reliable PoC coagulation analyser to diagnose coagulopathy	13,90-92

Dengue	◦Multiple commercial antigen and antibody tests (e.g. SD BIOLINE Dengue Duo Rapid Test Kit and ASSURE®Dengue IgA Rapid test (antigen-based)	◦Field-deployable PoC test without cross reactivity with the Zika virus ◦Novel circulating biomarker identification ◦Multiplexed test with the Zika virus	13,93-95
Dracunculiasis	◦ No commercial tests reported	◦Field-deployable nucleic-acid-based test for copepods (crustaceans) to test bodies of water for surveillance purposes during the current eradication era	8
Leprosy	◦1 biomarker test	◦Quality assessment and field validation	38
Soil-transmitted helminthiases	◦Kankanet (smart microscopy tool)	◦Improved algorithms for other infectious species as Kankanet can only detect *Ascaris lumbricoides* ◦Field-deployable test to detect resistance to treatment and test-of-cure ◦Multiplexed tests for other helminth infections	8,96
Trachoma	◦1 lateral flow test (antigen-based) ◦Grading tool	◦Quality assessment and field validation ◦PoC test with enhanced sensitivity to detect acute infections ◦Confirmatory test (test-of-cure)	97-99
Yaws	◦Dual Path Platform (DPP®)	◦Quality assessment and field-deployable nucleic-acid-based test to distinguish between yaws and syphilis ◦Multiplexed test with syphilis	100-103

Analysis of the status quo of NTDs diagnostics practices highlighted that, although technically adequate PoC tests might exist for certain NTDs, it is the disease unawareness and test unavailability in the endemic regions that often delimit the effective use of PoC tests. Henceforth, based on the WHO's 2021-2030 roadmap[8] and Diagnostic Technical Advisory Group for NTDs[20], and our comprehensive literature survey (SI), we further analyzed the NTDs for disease awareness, tests availability, and diagnostic technology insufficiencies (Fig 4). To rank the diagnostic insufficiencies of the 24 NTDs, we identified three parameters: awareness, availability, and diagnostic technology, and scored the needs for each NTD from 'in control' to 'action critically needed'. For many of the NTDs, we found that the major diagnostic insufficiency was contributed by test unavailability and lack of disease awareness, as seen for the 8 NTDs in the far left of Fig 4. For example, diagnosis of snakebite envenoming and rabies is delayed due to preference to traditional healing practices because there is lack of awareness and existing tests are not readily available. Similarly, for skin NTDs including, chromoblastomycosis, mycetoma and cutaneous leishmaniasis misdiagnosis due to lack of awareness results in delayed diagnosis and treatment. Likewise, diagnostic insufficiencies for foodborne trematodiases, echinococcosis, scabies, and other ectoparasites could be addressed by ensuring disease awareness and existing test availability, and novel PoC diagnostics should be developed only if efficient diagnosis is still not achieved. Summing up, it is pivotal to urgently address

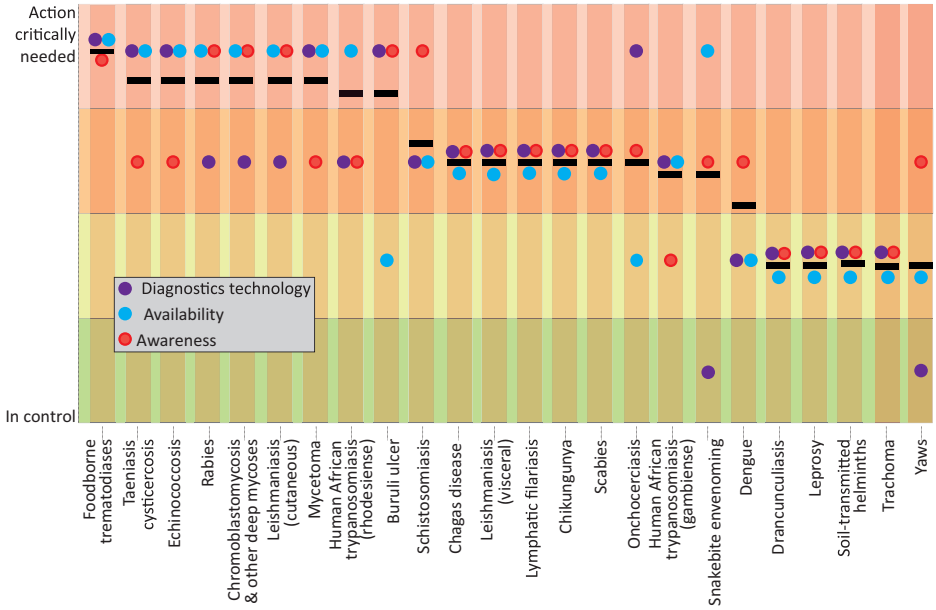

Figure 2.4: Diagnostic insufficiencies for neglected tropical diseases. NTDs were ranked in the order of diagnostic insufficiencies (black bars) as deduced from three parameters: diagnostics technology (purple), availability (blue), and awareness (red), and scored for 'action critically needed' to 'in control'. Information was obtained from a literature survey, including comprehensive recent reports from the WHO, particularly the WHO 2021-2030 roadmap[8], and the diagnostic technical advisory group for NTDs report[20]. Data were critically analyzed to gather diagnostic insufficiencies for individual diseases.

the issues of disease awareness and test availability because they act as the main obstacles in the introduction of novel PoC tests in endemic settings.

In addition to disease unawareness and tests unavailability, other limitations and barriers can hinder successful implementation of PoC tests for NTDs.[17,42] PoC tests can potentially yield inaccurate test results due to poor analytical quality or improper sample handling – indicating a need for staff training for proper test protocols, result documentation, and device maintenance. Furthermore, the clinical relevance of a PoC test over conventional reference diagnostic standards has been a persistent concern among medical practitioners. Since 'first generation' lateral-flow rapid immunochromatographic tests for infectious diseases were often questioned for their analytical quality over laboratory counterparts, new sophisticated PoC tests are often subjected to some mistrust by medical practitioners in remote regions.[43] Surprisingly, rapid on-the-spot results can in practice even be a disadvantage, especially for diseases that require post-test counselling by a medical practitioner. Examples are chagas disease (American trypanosomiasis) that can be transmitted sexually, skin NTDs that are associated with social stigma, or diseases that may cause life-long

physical disabilities such as an amputation of an affected body part in case of buruli ulcer. In such cases, a positive test result of a PoC test can significantly affect the mental and social well-being of the patient and the healthcare worker, necessitating training of healthcare workers to provide psychological support. Moreover, since diagnosis of NTDs is currently often based on physical examination and suspicion due to disease endemicity in the region, a negative PoC test result can challenge and jeopardize the relationship between the patient and the healthcare worker or the medical practitioner,[44] possibly even yielding more general mistrust towards western medicine and a shift of healthcare seeking behavior towards traditional healers.

Finally, there are issues of cost and political context. The implementation of PoC tests for NTDs should follow standardized clinical pathways to ensure the judicious use of the PoC test.[1] Lack of technical support and maintenance is often an issue, especially in remote settings. Since sophisticated research is needed for the development of PoC diagnostic tests, many tests are far from cost effective, especially for NTDs wherein all interventions are donor-dependent and generally administered free of charge. Furthermore, costs due to supply chain, transport, and storage can hinder the sustainable use of a PoC test for NTDs.[45] In general, health services in endemic regions are insufficiently equipped for the management of NTDs and a lack of governmental support to implement innovative PoC tests for NTDs can be challenging, especially when new PoC tests do not confer a direct economic advantage over existing laboratory-based diagnostic test in the region.[46]

2.4 Discussion and conclusion

From our review of the status quo of PoC diagnostic tests and practices for all of the 24 NTDs mentioned in the WHO's 2021-2030 roadmap, we determined the diagnostic needs and formulated prerequisites of the relevant PoC tests. We identified that confirmatory diagnosis and a test-of-cure are the most common implementation needs for all NTDs. A paradigm shift is needed towards developing fully automated, robust PoC tests for confirmatory NTDs diagnostics. Additionally, due to a vicious circle of disease endemicity, i.e., poor healthcare seeking behavior due to extreme poverty, lack of awareness, persistent migration, and poor livelihood of marginalized populations, NTD diagnostic tests must be brought to the doorsteps of remote communities. Field-deployable multiplexed PoC tests at the local community level for NTD screening would benefit treatment but also aid in reaching the desired goal of NTD elimination. Multiplexing diagnostics of related illnesses would not only ensure correct diagnoses, identifying comorbidities and benefitting timely treatment, but also eliminate problems due to misdiagnoses and reduce financial burdens upon seeking healthcare.

Developing technically advanced PoC diagnostic tests does not automatically en-

sure that they will be routinely used in endemic regions. Therefore, in addition to technically improving PoC diagnostic tests, it is vital that future interventions focus on creating awareness programs and developing appropriate logistics to ensure that the necessary PoC tests are available within endemic regions for those NTDs where the major diagnostic need lies in a lack of awareness and test unavailability. In other words, it is important to determine if a NTD requires a novel PoC test or if efforts should enhance awareness or increase availability of existing PoC tests. Research should thus only focus on developing novel PoC tests for those NTDs where the technical inefficiencies of existing diagnostic tests present the main barrier. Enhancing local awareness of the NTD involves progressive knowledge transfer and capacity building initiatives to engage and empower the local communities. Lessons learnt from successful diagnostic strategies should be customized and applied to NTDs. For example, the success stories from malaria control programs can be adapted to related febrile illnesses such as dengue and chikungunya.

Since multiple NTDs are often prevalent in endemic regions, common solutions can be combined and integrated. In almost every individual NTD study, we observed that complex multiparametric factors are present that involve challenges and potential strategies to combat them. For example, skin NTDs such as buruli ulcer, cutaneous leishmaniasis, leprosy, mycetoma, yaws, and onchocerciasis all require common diagnostics practices and case-management strategies[47] and thus can be integrated for disease awareness, disease mapping, and training of the healthcare workers. Likewise, linking logistics to ensure availability of the diagnostics tests in remote regions presents opportunities for integration, i.e., the supply chain for multiple tests can be combined so that the tests can be delivered together from national hospitals to the peripheral health facilities in the endemic regions.

Above and beyond all technicalities, NTD-afflicted communities have inadequate basic amenities such as water, food, housing, etc., which cause further spread of diseases in endemic regions. For example, water often plays a critical and common role in the spread of NTDs. Schistosomiasis is a waterborne disease, wherein parasites multiply in water snails, and hence water acts as a reservoir of this disease vector. Likewise, trachoma is a water-scarce disease, wherein person-to-person transmission is due to a lack of water for basic hygiene. Thus, for combating schistosomiasis and trachoma, a common solution could be to ensure the availability of quality water along with basic sanitation and hygiene practices (WASH).[48] Similarly, insect-transmitted diseases such as dengue, chikungunya, trypanosomiasis and leishmaniasis can be combatted together by integrating vector-control programs like insecticide-treated nets, indoor-insecticide spraying, treatment of water-reservoirs, and awareness programs. Hence, multiple NTDs that have direct co-relation with the basic needs such as clean water, sanitation, nutrition, vector-management activities, veterinary public health activities, etc., can be combated together by pro-

viding common solutions. In essence, instead of vertical interventions that target one disease at a time, multiple NTDs can be tackled simultaneously by horizontally integrating related interventions to achieve common objectives and thereby aid the prevention and control of NTDs.

Finally, for the successful control and desired eradication of an NTD, customized PoC diagnostic solutions need to be devised. The healthcare paradox in NTD endemic regions is that the highest need for healthcare occurs in areas with minimum resources, i.e., at the lowest level of healthcare system, thus posing significant challenges for patients that seek adequate healthcare (Fig 5). Thus, diagnostic solutions should not only be patient-centric and abide to context-specific requirements as highlighted above, but they should also be in line with subsequent treatment approaches and healthcare system in general. For example, if treatment requires hospitalization, a PoC diagnostic test for screening of the disease would suffice in the field and follow-up tests can be performed at the hospital. However, for NTDs wherein treatment can be limited to medication that can be administered at home or at a local minimally equipped healthcare center, a robust confirmatory PoC diagnostic test would be needed. In a nutshell, a context-specific holistic approach is necessitated that combines early disease diagnosis, effective treatment interventions, and control strategies for multiple diseases at the same time. [12,44-47]

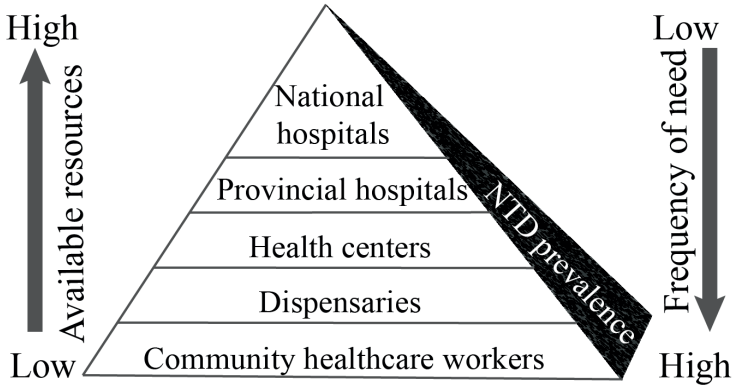

Figure 2.5: The healthcare paradox for NTDs in endemic regions. At the lowest level, the healthcare system in the NTD endemic region, has minimally trained community healthcare workers (CHWs) capable of providing referrals to a level-up such as local dispensaries with limited resources including technicians and/or midwives, followed by health centers with trained medical professionals, laboratory space, equipment and/or in-patient wards. One level further up are adequately equipped provisional hospitals followed by fully functional national hospitals at the top of the pyramid. Notably, the burden of NTDs is highest at the lowest healthcare level. This indicates the healthcare paradox, i.e., the highest NTD needs occur at the base of the pyramid where resources are inadequate, and thus the disease spreads further due to unmet healthcare needs. To interrupt this vicious cycle of disease endemicity, easy-to-use NTD diagnostics should be introduced to the CHWs at the lowest level. Bringing NTD diagnostics to the door-step of the endemic populations will ensure efficient disease diagnosis and treatment, reduce costs, and thereby alleviate the healthcare burden.

Summing up, to accelerate the global efforts for management of NTDs, cross-cutting strategies spanning across all NTDs interventions from the development of new PoC diagnostic tests to treatment and control, to improvements such as WASH, nutrition and living standards, to issues of cost and political context need to work hand-in-hand. Especially now that emerging diseases cause global pandemics, consolidated initiatives such as the "one health" approach[49] is fitting for the timely management of NTDs to alleviate the global healthcare burden.

2.5 Methods

A literature review was conducted using an exploratory search strategy of electronic databases, including Google Scholar, PubMed, Medline, Google Patents, Google Books, Web of science, Espacenet, Pubget, Scopus, IEEExplore, Open Content, WHO websites, CDC website, Wikipedia. For the entire review, we searched for research articles with the keywords relevant to each section of the review. We used MeSH (Medical Subject Headings) terms for disease names and causal species names, in combination with keywords including diagnostics, rapid diagnostic tests, commercial tests, point-of-care tests/testing, NTD diagnostics, bedside testing', 'remote rapid testing', 'near-patient laboratory testing', 'ancillary testing', and 'decentralized testing etc. in different combinations. We analyzed all articles and reports published and included those relevant to the scope of this review. Additional articles were obtained by citation tracking of review and original articles. For the comprehensive overview provided in the SI, NTDs were equally divided among the authors, (i.e. 4-5 NTDs per author) to produce an overview of the PoC diagnosis for each NTD (see SI), yielding a brief description of the disease, the current diagnostics, and PoC tests, if available. Thereafter, the overview of PoC diagnosis of the NTDs were reviewed by the first authors and implementation needs were deduced. Implementation needs refer to specific technical requirements that would support the translation from proof-of-principle tests to field-deployable PoC tests. Any discrepancies were discussed among all authors until a consensus was reached.

References

1. Standardization IO for. Point-of-care Testing (POCT): Requirements for Quality and Competence. 2006.
2. Luppa P, Junker R. Point-of-Care Testing, Principles and Clinical Applications. Springer; 2018. (Springer).
3. Diagnosing infections—current and anticipated technologies for point-of-care diagnostics and home-based testing. 2018.
4. Drain PK, Hyle EP, Noubary F, Freedberg KA, Wilson D, Bishai WR, et al. Diagnostic point-of-care tests in resource-limited settings. The Lancet Infectious Diseases. 2014 Mar;14(3):239–49.
5. Dincer C, Bruch R, Kling A, Dittrich PS, Urban GA. Multiplexed Point-of-Care Testing – xPOCT. Trends in Biotechnology. 2017 Aug 1;35(8):728–42.
6. Yang H, Chen Z, Cao X, Li Z, Stavrakis S, Choo J, et al. A sample-in-digital-answer-out system for rapid detection and quantitation of infectious pathogens in bodily fluids. Analytical and Bioanalytical Chemistry. 2018 Aug 29;366(5):1–12
7. Swinney DC, Pollastri MP. Neglected Tropical Diseases. John Wiley & Sons; 2019. (John Wiley & Sons).
8. WHO. Ending the neglect to attain the sustainable development goals: a road map for neglected tropical diseases 2021–2030. World Health Organization. 2020.
9. Mitra AK, Mawson AR. Neglected Tropical Diseases: Epidemiology and Global Burden. Tropical medicine and infectious disease. 2017 Aug 5;2(3):36.
10. Molyneux DH, Savioli L, Engels D. Neglected tropical diseases: progress towards addressing the chronic pandemic. Lancet. 2017;389(10066):312–25.
11. Hollingsworth TD. Counting Down the 2020 Goals for 9 Neglected Tropical Diseases: What Have We Learned From Quantitative Analysis and Transmission Modeling? Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2018 Jun 1;66(suppl_4):S237–44.
12. Kumar A. Picturing health: speak up, do more-the first World NTD Day. Lancet [Internet]. 2020 Feb 22;395(10224):551–8. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0140673620302567
13. WHO. WHO global consultations for new roadmap on NTDs [Internet]. 2019 [cited 1AD]. Available from: https://www.who.int/neglected_diseases/news/WHO-global-consultations-for-new-Roadmap-on-NTD/en/
14. Peeling RW, Holmes KK, Mabey D, Ronald A. Rapid tests for sexually transmitted infections (STIs): the way forward. Sex Transm Infect. 2006;82(suppl 5):v1.
15. Land KJ, Boeras DI, Chen X-S, Ramsay AR, Peeling RW. REASSURED diagnostics to inform disease control strategies, strengthen health systems and improve patient outcomes. Nat Microbiol. 2019;4(1):46–54.
16. Pai NP, Vadnais C, Denkinger C, Engel N, Pai M. Point-of-Care Testing for Infectious Diseases: Diversity, Complexity, and Barriers in Low- And Middle-Income Countries. PLoS Medicine [Internet]. 2012 Sep 4;9(9):e1001306-7. Available from:http://journals.plos.org/plosmedicine/article/file?id=10.1371/journal.pmed.1001306&type=printable
17. Shephard M. A Practical Guide to Global Point-of-Care Testing. CSIRO PUBLISHING; 2016. (CSIRO PUBLISHING).
18. Mabey D, Peeling RW, Ustianowski A, Perkins MD. Diagnostics for the developing world.

Nature Reviews Microbiology [Internet]. 2004 Mar 1;2(3):231–40. Available from: https://www.nature.com/articles/nrmicro841.pdf
19. Kost GJ, Tran NK, Louie RF. Point-of-Care Testing: Principles, Practice, and Critical-Emergency-Disaster Medicine. 8th ed. American Cancer Society; 2006. (American Cancer Society; vol. 104).
20. WHO. Report of the first meeting of the WHO Diagnostic Technical Advisory Group for Neglected Tropical Diseases [Internet]. 2020. Available from: https://apps.who.int/iris/bitstream/handle/10665/331954/9789240003590-eng.pdf?ua=1
21. Kozel TR, Burnham-Marusich AR. Point-of-care testing for infectious diseases: Past, present, and future. Kraft CS, editor. Journal of Clinical Microbiology. 2017 Aug 1;55(8):2313–20.
22. Campbell S, Landry ML. Rapid antigen tests. In: 9th ed. 2014. p. 31–51. (Advanced Techniques in Diagnostic Microbiology; vol. 71).
23. Lode P von. Point-of-care immunotesting: Approaching the analytical performance of central laboratory methods. Clinical Biochemistry. 2005 Jul;38(7):591–606.
24. Carter IWJ, Schuller M, James GS, Sloots TP, Halliday CL. PCR for Clinical Microbiology. Springer Science & Business Media; 2010. (Springer Science & Business Media).
25. Butler J. Advanced Topics in Forensic DNA Typing. Elsevier Inc.; 2012. (Advanced Topics in Forensic DNA Typing; vol. 1).
26. Ferguson T, Niemz A. Nucleic Acid Isothermal Amplification Technologies and Point-of-Care Diagnostics. In Pan Stanford; 2014. p. 409–26. (Viral Diagnostics; vol. 276).
27. Craw P, Balachandran W. Isothermal nucleic acid amplification technologies for point-of-care diagnostics: a critical review. Lab on a Chip. 2012 Jun 19;12(14):2469–86.
28. Kellner MJ, Koob JG, Gootenberg JS, Abudayyeh OO, Zhang F. SHERLOCK: nucleic acid detection with CRISPR nucleases. Nature Protocols. 2019 Oct 1;14(10):2986–3012.
29. Myhrvold C, Freije CA, Gootenberg JS, Abudayyeh OO, Metsky HC, Durbin AF, et al. Field-deployable viral diagnostics using CRISPR-Cas13. Science. 2018 Apr 27;360(6387):444–8.
30. Chertow DS. Next-generation diagnostics with CRISPR. Science. 2018 Apr 27;360(6387):381–2.
31. Li Y, Li S, Wang J, Liu G. CRISPR/Cas Systems towards Next-Generation Biosensing. Trends in Biotechnology. 2019 Jan 14;
32. Chiu C. Cutting-Edge Infectious Disease Diagnostics with CRISPR. Cell Host & Microbe. 2018 Jun 13;23(6):702–4.
33. Tang YW, Stratton CW. Advanced techniques in diagnostic microbiology. Tang YW, Stratton CW, editors. Springer International Publishing; 2018. (Advanced Techniques in Diagnostic
34. Minh VD, Hanh LQ, Vu M. Clinical use of nucleic-acid-amplification tests. Chest. 2000 Jan 1;118(3):574–5.
35. Furrows SJ, Ridgway GL. "Good laboratory practice" in diagnostic laboratories using nucleic acid amplification methods. Clinical Microbiology and Infection. 2001 May;7(5):227–9.
36. Cantera JL, White H, Diaz MH, Beall SG, Winchell JM, Lillis L, et al. Assessment of eight nucleic acid amplification technologies for potential use to detect infectious agents in low-resource settings. Kalendar R, editor. PLoS One. 2019;14(4):e0215756.
37. Aabenhus R, Jensen J-US, Jørgensen KJ, Hróbjartsson A, Bjerrum L. Biomarkers as point-

of-care tests to guide prescription of antibiotics in patients with acute respiratory infections in primary care. Group CARI, editor. Cochrane Database of Systematic Reviews. 2014 Nov

38. Hooij A van, Eeden S van den, Richardus R, Fat ETK, Wilson L, Franken KLMC, et al. Application of new host biomarker profiles in quantitative point-of-care tests facilitates leprosy diagnosis in the field. Ebiomedicine. 2019 Sep;47:301–8.
39. Reali S, Najib EY, Balázs KET, Tan ACH, Váradi L, Hibbs DE, et al. Novel diagnostics for point-of-care bacterial detection and identification. RSC Advances. 2019;9(37):21486–97.
40. Yadav GS, Kumar V, Aggarwal NK. Aptamers. Springer Nature; 2019. (Springer Nature).
41. Pan Q, Luo F, Liu M, Zhang XL. Oligonucleotide aptamers: promising and powerful diagnostic and therapeutic tools for infectious diseases. Journal of Infection. 2018 Aug 1;77(2):83–98.
42. Shaw JLV. Practical challenges related to point of care testing. Practical Laboratory Medicine. 2016 Apr 1;4:22–9.
43. John AS. The Evidence to Support Point-of-Care Testing. The Clinical biochemist Reviews. 2010 Aug;31(3):111–9.
44. Hays JP, Mitsakakis K, Luz S, Belkum A van, Becker K, Bruel A van den, et al. The successful uptake and sustainability of rapid infectious disease and antimicrobial resistance point-of-care testing requires a complex "mix-and-match" implementation package. European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology. 2019 Jun;38(6):1015–22.
45. Hicks JM, Haeckel R, Price CP, Lewandrowski K, Wu A. Recommendations and opinions for the use of point-of-care testing for hospitals and primary care: summary of a 1999 symposium. Clinica Chimica Acta. 2001 Jan;303(1–2):1–17.
46. Freedman DB. Clinical governance: implications for point-of-care testing. Annals of clinical biochemistry [Internet]. 2002 Sep;39(Pt 5):421–3. Available from: http://acb.sagepub.com/lookup/doi/10.1258/000456302320314421
47. WHO. RECOGNIZING NEGLECTED TROPICAL DISEASES THROUGH CHANGES ON THE SKIN [Internet]. 2018 [cited 2020 Nov 6]. Available from: http://apps.who.int/iris/bitstream/handle/10665/272723/9789241513531-eng.pdf?ua=1
48. WHO. Water Sanitation & Hygiene 2015–2020 A GLOBAL STRATEGY for accelerating and sustaining progress on Neglected Tropical Diseases [Internet]. n.d. Available from: http://apps.who.int/iris/bitstream/10665/182735/1/WHO_FWC_WSH_15.12_eng.pdf?
49. WHO. The Control of Neglected Zoonotic Diseases [Internet]. 2015 Jul p. 1–48. (World Health Organization). Available from: https://www.who.int/neglected_diseases/ISBN9789241508568_ok.pdf?ua=1
50. Mubanga C, Mwape KE, Phiri IK, Trevisan C, Zulu G, Chabala C, et al. Progress on the development of rapid diagnostic tests for foodborne neglected zoonotic helminthiases: A systematic review. Acta Tropica. 2019 Jun;194:135–47.
51. Li L, Liu X, Zhou B, Zhang S, Wang G, Ma G, et al. Multiple food-borne trematodiases with profound systemic involvement: a case report and literature review. BMC Infectious Diseases. 2019 Jun 14;19(1):526.
52. Sadaow L, Sanpool O, Rodpai R, Yamasaki H, Ittiprasert W, Mann VH, et al. Development of an Immunochromatographic Point-of-Care Test for Serodiagnosis of Opist-

horchiasis and Clonorchiasis. The American Journal of Tropical Medicine and Hygiene. 2019;101(5):1156–60.
53. Organization WH. Taenia solium taeniasis/cysticercosis diagnostic tools: report of a stakeholder meeting, Geneva, 17-18 December 2015. 2016. (World Health Organization).
54. Corstjens PLAM, Dood CJ de, Priest JW, Tanke HJ, Handali S, in PCWG. Feasibility of a lateral flow test for neurocysticercosis using novel up-converting nanomaterials and a lightweight strip analyzer. Gabriël S, editor. PLOS Neglected Tropical Diseases [Internet]. 2014 Jul;8(7):e2944–e2944. Available from: https://pubmed.ncbi.nlm.nih.gov/24992686
55. Tamarozzi F, Covini I, Mariconti M, Narra R, Tinelli C, Silvestri AD, et al. Comparison of the Diagnostic Accuracy of Three Rapid Tests for the Serodiagnosis of Hepatic Cystic Echinococcosis in Humans. Hirayama K, editor. PLOS Neglected Tropical Diseases. 2016 Feb 1;10(2):e0004444.
56. Certoma A, Lunt RA, Vosloo W, Smith I, Colling A, Williams DT, et al. Assessment of a rabies virus rapid diagnostic test for the detection of Australian bat lyssavirus. Tropical medicine and infectious disease. 2018 Oct 4;3(4):109.
57. Léchenne M, Naïssengar K, Lepelletier A, Alfaroukh IO, Bourhy H, Zinsstag J, et al. Validation of a Rapid Rabies Diagnostic Tool for Field Surveillance in Developing Countries. Rupprecht CE, editor. PLOS Neglected Tropical Diseases. 2016 Oct;10(10):e0005010.
58. Kang B, Oh J, Lee C, Park B-K, Park Y, Hong K, et al. Evaluation of a rapid immunodiagnostic test kit for rabies virus. Journal of virological methods. 2007 Oct;145(1):30–6.
59. Bienvenu A-L, Picot S. Mycetoma and Chromoblastomycosis: Perspective for Diagnosis Improvement Using Biomarkers. Molecules. 2020 Jan 1;25(11):2594.
60. Schallig HDFH, Hu RVP, Kent AD, Loenen M van, Menting S, Picado A, et al. Evaluation of point of care tests for the diagnosis of cutaneous leishmaniasis in Suriname. BMC Infectious Diseases. 2019 Dec 1;19(1):25.
61. Veland N, Espinosa D, Valencia BM, Ramos AP, Calderon F, Arevalo J, et al. Polymerase chain reaction detection of Leishmania kDNA from the urine of Peruvian patients with cutaneous and mucocutaneous leishmaniasis. The American Journal of Tropical Medicine and Hygiene [Internet]. 2011 Apr;84(4):556–61. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3062448/pdf/tropmed-84-556.pdf
62. Adams ER, Schoone G, Versteeg I, Gomez MA, Diro E, Mori Y, et al. Development and Evaluation of a Novel Loop-Mediated Isothermal Amplification Assay for Diagnosis of Cutaneous and Visceral Leishmaniasis. Loeffelholz MJ, editor. Journal of Clinical Microbiology. 2018 Jul;56(7):S102.
63. Mirzaei A. Immunodetection and molecular determination of visceral and cutaneous Leishmania infection using patients' urine. Infection, Genetics and Evolution. 2018 Sep 1;63:257–68.
64. DNDi. Mycetoma: New hope for neglected patients - Developing effective treatments for a truly neglected disease [Internet]. 2019. Available from: https://www.dndi.org/wp-content/uploads/2019/02/DNDi_Mycetoma_2019.pdf
65. Bonnet J, Boudot C, Courtioux B. Overview of the Diagnostic Methods Used in the Field for Human African Trypanosomiasis: What Could Change in the Next Years? BioMed Research International. 2015;2015(1):583262–10.
66. Biéler S, Waltenberger H, Barrett MP, McCulloch R, Mottram JC, Carrington M, et al.

Evaluation of Antigens for Development of a Serological Test for Human African Trypanosomiasis. Tanowitz HB, editor. PLoS One. 2016;11(12):e0168074.
67. Matovu E, Kazibwe AJ, Mugasa CM, Ndung'u JM, Njiru ZK. Towards Point-of-Care Diagnostic and Staging Tools for Human African Trypanosomiaisis. Journal of tropical medicine. 2012;2012(4):340538–9.
68. Sakyi SA, Aboagye SY, Otchere ID, Yeboah-Manu D. Clinical and Laboratory Diagnosis of Buruli Ulcer Disease: A Systematic Review. The Canadian journal of infectious diseases & medical microbiology = Journal canadien des maladies infectieuses et de la microbiologie medicale. 2016;2016(17):5310718–10.
69. Organization WH. Integrating Neglected Tropical Diseases in Global Health and Development: Fourth Who Report on Neglected Tropical Diseases. 2018.
70. Diesburg SP, Guelig D, Burton R, Singleton J, Labarre P. Development of a single-use, disposable, electricity-free, nucleic acid amplification platform. GHTC. 2015;400–6.
71. Utzinger J, Becker SL, Knopp S, Blum J, Neumayr AL, Keiser J, et al. Neglected tropical diseases: diagnosis, clinical management, treatment and control. Swiss medical weekly. 2012;142(4748):w13727.
72. Legesse M, Erko B. Field-based evaluation of a reagent strip test for diagnosis of Schistosoma mansoni by detecting circulating cathodic antigen in urine before and after chemotherapy. Transactions of the Royal Society of Tropical Medicine and Hygiene. 2007 Jul;101(7):668–73.
73. Colley DG, King CH, Kittur N, Ramzy RMR, Secor WE, Fredericks-James M, et al. Evaluation, Validation, and Recognition of the Point-of-Care Circulating Cathodic Antigen, Urine-Based Assay for Mapping Schistosoma mansoni Infections. Am J Tropical Medicine Hyg. 2020;103(1_Suppl):42–9.
74. Beltrame A, Guerriero M, Angheben A, Gobbi F, Requena-Mendez A, Zammarchi L, et al. Accuracy of parasitological and immunological tests for the screening of human schistosomiasis in immigrants and refugees from African countries: An approach with Latent Class Analysis. Plos Neglect Trop D. 2017;11(6):e0005593.
75. Shah V, Ferrufino L, Gilman RH, Ramirez M, Saenza E, Malaga E, et al. Field Evaluation of the InBios Chagas Detect Plus Rapid Test in Serum and Whole-Blood Specimens in Bolivia. Wilkins PP, editor. Clinical and vaccine immunology : CVI. 2014 Dec;21(12):1645–9.
76. Ponce C, Ponce E, Vinelli E, Montoya A, Aguilar V de, Gonzalez A, et al. Validation of a Rapid and Reliable Test for Diagnosis of Chagas' Disease by Detection of Trypanosoma cruzi-Specific Antibodies in Blood of Donors and Patients in Central America. Journal of Clinical Microbiology. 2005 Oct 1;43(10):5065–8.
77. Boelaert M, Verdonck K, Menten J, Sunyoto T, Griensven J van, Chappuis F, et al. Rapid tests for the diagnosis of visceral leishmaniasis in patients with suspected disease. Group CID, editor. Cochrane Database of Systematic Reviews. 2014;2014(6).
78. Singh OP, Sundar S. Developments in diagnosis of visceral leishmaniasis in the elimination era. Journal of Parasitology Research. 2015 Jan 1;2015:239469.
79. Mondal D, Ghosh P, Khan MAA, Hossain F, Böhlken-Fascher S, Matlashewski G, et al. Mobile suitcase laboratory for rapid detection of Leishmania donovani using recombinase polymerase amplification assay. Parasites & Vectors. 2016 May 13;9(1):281–8.
80. Rebollo MP, Bockarie MJ. Can Lymphatic Filariasis Be Eliminated by 2020? Trends in Parasitology. 2017 Feb;33(2):83–92.
81. Hertz MI, Nana-Djeunga H, Kamgno J, Njouendou AJ, Chunda VC, Wanji S, et al. Iden-

tification and characterization of Loa loa antigens responsible for cross-reactivity with rapid diagnostic tests for lymphatic filariasis. Specht S, editor. PLOS Neglected Tropical Diseases [Internet]. 2018 Nov;12(11):e0006963. Available from:http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=30444866&retmode=ref&cmd=prlinks

82. Hayashida K, Orba Y, Sequeira PC, Sugimoto C, Hall WW, Eshita Y, et al. Field diagnosis and genotyping of chikungunya virus using a dried reverse transcription loop-mediated isothermal amplification (LAMP) assay and MinION sequencing. Kittayapong P, editor. PLOS Neglected Tropical Diseases. 2019 Jun 1;13(6):e0007480.
83. Vongsouvath M, Bharucha T, Seephonelee M, Lamballerie X de, Newton PN, Dubot-Pérès A. Harnessing Dengue Rapid Diagnostic Tests for the Combined Surveillance of Dengue, Zika, and Chikungunya Viruses in Laos. The American Journal of Tropical Medicine and Hygiene. 2020 Jun;102(6):1244–8.
84. Kinyanjui T, Middleton J, Güttel S, Cassell J, Ross J, House T. Scabies in residential care homes: Modelling, inference and interventions for well-connected population subunits. Alizon S, editor. PLoS computational biology. 2018 Mar;14(3):e1006046.
85. Leung V, Miller M. Detection of Scabies: A Systematic Review of Diagnostic Methods. Canadian Journal of Infectious Diseases and Medical Microbiology [Internet]. 2011;22(4):143–6. Available from: https://www.hindawi.com/journals/cjidmm/2011/698494/
86. Park JH, Kim CW, Kim SS. The diagnostic accuracy of dermoscopy for scabies. Annals of dermatology. 2012 May;24(2):194–9.
87. Steel C, Golden A, Stevens E, Yokobe L, Domingo GJ, Santos T de los, et al. Rapid Point-of-Contact Tool for Mapping and Integrated Surveillance of Wuchereria bancrofti and Onchocerca volvulus Infection. Wilkins PP, editor. Clinical and vaccine immunology : CVI. 2015 Aug;22(8):896–901.
88. Lumbala C, Biéler S, Kayembe S, Makabuza J, Ongarello S, Ndung'u JM. Prospective evaluation of a rapid diagnostic test for Trypanosoma brucei gambiense infection developed using recombinant antigens. Caljon G, editor. PLOS Neglected Tropical Diseases. 2018 Mar 28;12(3):e0006386.
89. Boelaert M, Mukendi D, Bottieau E, Lilo JRK, Verdonck K, Minikulu L, et al. A Phase III Diagnostic Accuracy Study of a Rapid Diagnostic Test for Diagnosis of Second-Stage Human African Trypanosomiasis in the Democratic Republic of the Congo. Ebiomedicine. 2018 Jan 1;27:11–7.
90. Williams HF, Layfield HJ, Vallance T, Patel K, Bicknell AB, Trim SA, et al. The Urgent Need to Develop Novel Strategies for the Diagnosis and Treatment of Snakebites. Toxins. 2019 Jun 20;11(6):363.
91. Laxme RRS, Khochare S, Souza HF de, Ahuja B, Suranse V, Martin G, et al. Beyond the "big four": Venom profiling of the medically important yet neglected Indian snakes reveals disturbing antivenom deficiencies. BILLIALD P, editor. PLOS Neglected Tropical Diseases. 2019 Dec;13(12):e0007899.
92. O'Rourke KM, Correlje E, Martin CL, Robertson JD, Isbister GK. Point-of-care derived INR does not reliably detect significant coagulopathy following Australian snakebite. Thrombosis research. 2013 Nov;132(5):610–3.
93. Gan W, Gu Y, Han J, Li CX, Sun J, Liu P. Chitosan-Modified Filter Paper for Nucleic Acid Extraction and "in Situ PCR" on a Thermoplastic Microchip. Analytical Chemistry. 2017

Mar 21;89(6):3568–75.
94. Wang SM, Sekaran SD. Early diagnosis of Dengue infection using a commercial Dengue Duo rapid test kit for the detection of NS1, IGM, and IGG. The American Journal of Tropical Medicine and Hygiene. 2010 Sep;83(3):690–5.
95. Blacksell SD, Jarman RG, Bailey MS, Tanganuchitcharnchai A, Jenjaroen K, Gibbons RV, et al. Evaluation of six commercial point-of-care tests for diagnosis of acute dengue infections: the need for combining NS1 antigen and IgM/IgG antibody detection to achieve acceptable levels of accuracy. Clinical and vaccine immunology : CVI. 2011 Dec;18(12):2095–101.
96. Yang A, Bakhtari N, Langdon-Embry L, Redwood E, Lapierre SG, Rakotomanga P, et al. Kankanet: An artificial neural network-based object detection smartphone application and mobile microscope as a point-of-care diagnostic aid for soil-transmitted helminthiases. Bennuru S, editor. PLOS Neglected Tropical Diseases. 2019 Aug;13(8):e0007577.
97. Gwyn S, Mitchell A, Dean D, Mkocha H, Handali S, Martin DL. Lateral flow-based antibody testing for Chlamydia trachomatis. Journal of immunological methods. 2016 Aug;435:27–31.
98. Solomon AW, Mesurier RTL, Williams WJ. A diagnostic instrument to help field graders evaluate active trachoma. Ophthalmic epidemiology. 2018 Dec;25(5–6):399–402.
99. Harding-Esch EM, Holland MJ, Schémann J-F, Molina S, Sarr I, Andreasen AA, et al. Diagnostic accuracy of a prototype point-of-care test for ocular Chlamydia trachomatis under field conditions in The Gambia and Senegal. Schachter J, editor. PLOS Neglected Tropical Diseases. 2011 Aug;5(8):e1234.
100. Marks M, Goncalves A, Vahi V, Sokana O, Puiahi E, Zhang Z, et al. Evaluation of a rapid diagnostic test for yaws infection in a community surveillance setting. Phillips RO, editor. PLOS Neglected Tropical Diseases. 2014 Sep;8(9):e3156.
101. Chi K-H, Danavall D, Taleo F, Pillay A, Ye T, Nachamkin E, et al. Molecular Differentiation of Treponema pallidum Subspecies in Skin Ulceration Clinically Suspected as Yaws in Vanuatu Using Real-Time Multiplex PCR and Serological Methods. The American Journal of Tropical Medicine and Hygiene. 2015 Jan;92(1):134–8.
102. Yin Y-P, Chen X-S, Wei W-H, Gong K-L, Cao W-L, Yong G, et al. A Dual Point-of-Care Test Shows Good Performance in Simultaneously Detecting Nontreponemal and Treponemal Antibodies in Patients With Syphilis: A Multisite Evaluation Study in China. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2013;56(5):659–65.
103. Basing LAW, Simpson SV, Adu-Sarkodie Y, Linnes JC. A Loop-Mediated Isothermal Amplification Assay for the Detection of Treponema pallidum subsp. pertenue. The American Journal of Tropical Medicine and Hygiene [Internet]. 2020 Apr 20;tpmd190243. Available from: http://www.ajtmh.org/content/journals/10.4269/ajtmh.19-0243

2.6 Supplementary Information

This section contains an overview of all 24 neglected tropical diseases (NTDs) identified in the WHO's 2021-2030 roadmap, based on diagnostic requirements (an extension of the commonly used list of 20 NTDs). We present the NTDs in the order of their diagnostic insufficiencies (Figure 4) wherein for each NTD, we provide a brief one-page overview with

- A brief introduction with a description of the disease,
- Current diagnostics,
- Point-of-care (PoC) test, if available [note that 'antigen-based tests' use antigens in the test to detect antibodies in a sample. This contrasts 'antibody-based tests' that use antibodies in a test to detect antigens in a sample]
- Implementation need.

In the description below, the NTDs appear in the same order as for the figures and the table in the main manuscript.

2.6.1 Foodborne trematodiases

Foodborne trematodiases are caused by infections with trematode worms. Infectious species are *Clonorchis sinensis, Opisthorchis viverrini, Opisthorchis felineus, Fasciola hepatica, Fasciola gigantica*, and *Paragonimus species*.[1] Foodborne trematodiases are transmitted through raw and undercooked food (fish, aquatic vegetables, crabs and crayfish) that are infected with trematode larvae. Infection with trematode worms causes severe abdominal pain, malaise, inflammation and fibrosis of the liver, possibly fatal bile duct cancer (clonorchiasis and opisthorchiasis), colic pain and jaundice from fascioliasis (liver fluke infection), chronic cough and chest pain, shortness of breath (dyspnea), and fever from paragonimiasis (lung fluke infection). Foodborne trematodiases are found throughout the world, however, African and South East Asian countries have the highest burden. Approximately 200,000 people are infected annually, causing ~7,000 deaths and ~2 million DALYs (2016).[2] Children under the age of five account for approximately one-third of deaths. Foodborne trematodiases are treated by mass drug administration (MDA) of anthelminthic medication, such as praziquantel and triclabendazole, in endemic areas.[3] These infections are of great economic and public health importance, especially veterinary significance as livestock are affected as well.[4]

Current diagnostics

Diagnostics for foodborne trematodiases is done through imaging (X-ray, magnetic resonance imaging (MRI)), serology (ELISA), histopathology (definitive individual diagnosis) and parasitological microscopy techniques,[1] such as the detection of eggs in stool samples using the Kato-Katz thick smears method.[4,5] Polymerase chain reaction (PCR) tests are routinely used, especially in resource-limited settings.[4] Although microscopy is mainly used for diagnoses, it has low sensitivity and requires an experienced user.

PoC test

A few PoC tests have been developed for foodborne trematodiases.[4] There are two antigen-based tests and one nucleic acid-based test for fascioliasis, three nucleic acid-based tests for opisthorchis, one antibody test for paragonimiasis, and one antibody and one nucleic acid-based test for clonorchiasis.[4] However, many of these PoC tests have not been validated in the field, and are thus not routinely used. Furthermore, the DNA extraction step for these tests are limited to laboratories. There is an immunochromatography test (ICT) kit for *Clonorchis sinensis* and *Opisthorchis viverrini* that detects IgG antibodies, and does not require any equipment, but it still needs to be validated in the field before it can be routinely used.[6]

Implementation need

These PoC tests need to be validated in field in order for them to be routinely used in endemic regions. Current field deployable tests fail due to ineffective sample preparation (i.e. not cracking the eggs to release the DNA) which leads to false negative test results.[1] There is an urgent need for sample preparation methods that do not rely on the use of a laboratory.[4]

2.6.2 Taeniasis/cysticercosis

Taeniasis is an intestinal infectious disease that is caused by adult tapeworms from the genus *Taenia*. Cysticercosis is a particular taeniasis infection that is caused by *Taenia solium* (pork tapeworm). Among all the tapeworm infections, *T*. *solium* infections cause the most significant health issues. Infection with *T. solium* occurs when raw infected pork is eaten, via the fecal-oral route or via ingesting contaminated water. Most infected patients are asymptotic or presented with a mild headache. Although the larval cystic stage is relatively harmless in most tissues, the cysts can cause neurocysticercosis (NCC) when they develop in the nervous system. The brain and eye tissues are particularly susceptible to the formation of these cysts.[7] The most frequent symptoms of NCC are epilepsy and seizures, intracranial hypertension, hydrocephalus, vascular damage and stroke, cognitive deficit and depression.8 Cysticercosis is endemic in low income countries with poor sanitation and domestic pig raising in Africa, Asia, and Latin America. Approximately 5.5 million people are infected worldwide, causing ~28,000 deaths, and ~2.8 million DALYs. Taeniasis is treated by MDA of anthelminthic medication, such as niclosamide or praziquantel as a single dose, or albendazole for 3 days.[9]

Current diagnostics

Diagnosis of NCC is based on neuroimaging supported by immunological diagnosis.[7,8] Neuroimaging via computer tomography (CT) and magnetic resonance imaging (MRI) show the presence, number, location, and size of the *T. solium* tapeworms in the brain. MRI provides better images of small lesions and CT scans show the calcification in the brain that is caused by the parasite, indicating that preferably both MRI and CT tests should be used. However only CT scans are used in poor endemic countries.[8] Patients with a viable cyst infection test positive, while patients that only have calcified lesions test negative when using immunological diagnostic tests. However, cross reactions with antibodies of other parasites requires the use of immunological diagnostic tests in combination with neuroimaging.[8,10] Molecular techniques based on PCR to detect *T. solium* specific genes have also been developed, although they are not routinely used in endemic regions, as PCR testing is not suitable in the resource-limited settings.

PoC test

There are currently no commercial PoC diagnostic tests available for taeniasis.[11] There is a proof-of-principle immunological antigen-based lateral flow test (i.e. that detects antibodies in a sample) with high sensitivity and specificity. However, the antigens that are used in this test are difficult to produce.[12]

Implementation need

Novel biomarker identification and a newly developed PoC test (which could be biomarker or nucleic-acid-based) are urgently needed to contribute to the effective diagnosis of NCC in endemic regions.

2.6.3 Echinococcosis

Echinococcosis is a zoonotic parasitic disease that occurs in humans in two main clinical forms, cystic echinococcosis (CE) and alveolar echinococcosis (AE),[13] which are caused by the tapeworms *Echinococcus granulosus* and *Echinococcus multilocularis*, respectively.[14] Adult worms develop in the intestines of dogs, foxes, and other carnivores. The eggs are then ingested by humans via contaminated food, and larvae develop in organs such as the liver, lungs, kidneys, spleen, bones and muscle tissue. Both forms of this disease first develop asymptomatically as incubation periods can last for many years, until the larvae develop and trigger clinical symptoms, which can be fatal when left untreated. Chronic symptoms include cough, chest pain, and shortness of breath. Symptoms depend on the location of the cysts.[14] Echinococcosis is distributed in every continent except Antarctica and more than 1 million people are infected worldwide. The highest prevalence occurs in rural areas where cattle are slaughtered. The exact burden of this disease is unknown, however, the WHO reports that there are approximately 871,000 DALYs associated with this disease.[2] Echinococcosis is of both public health and economic importance, as infected cattle have decreased milk production, reduced fertility, and decrease in hide value. Echinococcosis is expensive and complicated to treat, as surgery and chemotherapy are often required. The cysts can be removed with the PAIR surgical technique (Puncture, Aspiration, Injection, Re-aspiration).[15]

Current diagnostics

Ultrasonography imaging is the reference standard for the diagnosis of both clinical forms of echinococcosis in humans. This is complemented with MRI and CT scans. Histopathology (definitive individual diagnosis) and molecular assays (PCR) can also be used. Serological tests are also able to detect antigens to support the imaging diagnosis.[15]

PoC test

There are several commercial immunological PoC tests (antigen-based) for both

clinical forms of this diseases, examples include VIRAPID®HYDRATIDOSIS, ADAMU-CE and RIDASCREEN®Echinococcus IgG test 16, and one LAMP (Loop-mediated isothermal amplification) detection test (NADH 1-LAMP) has also been developed for CE.[4] The current PoC tests should only be used to complement imaging diagnosis as they have poor sensitivity in detecting inactive cysts.

Implementation need

More reliable PoC tests are needed that can serve as test-of-cure for humans (antigen or nucleic-acid-based). Field-deployable PoC tests for dogs would also contribute to the control of this disease.[16]

2.6.4 Rabies

Rabies is caused by the rabies virus (RABV) and by other lyssaviruses. The bite of rabid animals (especially dogs) and the saliva of the infected host are mainly responsible for the transmission of rabies. Wildlife like raccoons, skunks, bats, and foxes are major reservoirs for rabies. The incubation period is highly variable from 2 weeks to 6 years (average 2–3 months). Rabies can cause progressive and possibly fatal neurological impairment. It affects all warm-blooded animals and the disease is endemic in many countries except on islands such as Australia and Antarctica. The WHO reports 60,000 deaths (2015) and ~1.6 million DALYs (2016) due to rabies. Vaccination can be done in endemic areas. Treatment includes a fast-acting rabies injection (rabies immune globulin) that needs to be administered as soon as possible after the bite to prevent the virus from spreading throughout the body. Once patients are symptomatic, there is a 99% mortality rate. Being a major zoonosis, the precise and rapid diagnosis of rabies is important for early treatment, effective prevention, and control measures.[17-23]

Current diagnostics

Traditional sellers staining test (SST) and histopathological methods are still in use for the diagnosis of rabies. Direct immunofluorescent test (dFAT) (antibody-based test) is the reference standard test for diagnosis of rabies in fresh brain tissues of dog, as recommended by both the World Organization for Animal Health (OIE) and the WHO. The mouse inoculation test (MIT) and PCR are superior methods that are also used for routine diagnosis. While the CDC has developed a PCR-based test for rabies, its use is not suitable in resource-limited settings.[20]

PoC test

There are multiple immunological PoC tests for dogs. [21,24,25] Commercially available tests include Vet-o-test Rabies Ag, Anigen Rapid Rabies Ag Test kit, Quicking Pet Rapid Test, Rapid Rabies Ag Test Kit, Rabies Virus AgRapid test and the VET Rabies Antigen Rapid test.

Implementation need

The major challenge for rabies diagnosis is the use of invasive samples such as post-mortem brain tissue samples. A non-invasive sample collection method is urgently needed for use ante-mortem. Hence, there is an urgent need for the identification of circulating biomarkers. A field-deployable test for humans, that is suitable for use in primary healthcare centres will be important upon the identification of novel circulating biomarkers.

2.6.5 Chromoblastomycosis

Chromoblastomycosis (CBM) is a chronic fungal dermatosis of the skin and subcutaneous tissue caused by species of melanized fungi of the family *Herpotrichiellaceae,* which is present in soil, plants, decomposing wool, and decomposing organic matter. The most common species are *Fonsecaea pedrosoi, Cladophialophora carrionii* and *Phialophora verrucose.* CBM is usually an occupational related disease that is predominant among agricultural workers, miners, and woodsmen aged between 20 to 60 years. It causes lesions on the limbs, face and neck with no direct evidence of human-to-human or animal-to-human transmission. The infection starts as etiologic (disease-causing) agents enter the body through punctured wounds. While the initial lesion is similar to a small pink rash, over time (~1 week) it develops into a polymorphic clinical appearance simulating various infectious and non-infectious diseases. The exact burden of this group of diseases is unknown.[26] CBM is a debilitating disease that is difficult to treat, and often leads to secondary bacterial infections, which poses a therapeutic challenge to clinicians. The treatment also varies in accordance to the severity of the disease and the patient's immune response ranging from chemotherapy to combination therapy. Severe lesions respond slowly and are sometimes resistant to anti-fungal drugs. Oral antifungal therapy is not effective. CBM has been reported to co-exist with other diseases including leishmaniasis and leprosy. Co-infections reduce the efficacy of the treatment and result in prolonged sickness.[27]

Current diagnostics

Diagnosis of CBM is based on direct microscopic examination (wet mount), followed by culturing of the biopsy to determine the most effective treatment options. Growth of fungi can take up to 6 weeks, and although the genus can be identified from culture, further species determination requires DNA sequencing methods. Recently, molecular diagnostics using PCR targeting ribosomal DNA of *Fonsecaea species* and a specific DNA segment for the identification of *Cladophialohora carrionii* have been developed as a laboratory-based test. An ELISA assay using the *C. carrionii* antigen (AgSPP) has also been developed, but this is still not available in the endemic regions. The current diagnosis requires trained personnel and an equipped laboratory, and new rapid diagnostic tests are urgently needed.[27,28]

PoC test
There are currently no commercial PoC tests for CMB.

Implementation need
A PoC test with the capabilities to determine species specificity is required, as therapeutic success is directly related to the causative agent.[29]

2.6.6 Cutaneous leishmaniasis
Cutaneous leishmaniasis (CL) is the most common form of leishmaniasis that is caused by ~20 different *Leishmania* (*L*) species, with *L. major* and *L.tropica* causing severe CL. CL accounts for 260,000 DALYs.[2] Symptoms include debilitating skin lesions (ulcers) that leave lifelong scars, serious disability, and stigma due to mutilation. CL does not have a specific endemic area. However, 85 % of new cases occur in Afghanistan, Algeria, Bolivia, Brazil, Colombia, Iran, Iraq, Pakistan, the Syrian Arab Republic and Tunisia.[2] CL can be treated with pentavalent antimonials, amphotericin B, pentamidine isethionate, paromomycin, and antifungals.[31]

Current diagnostics
CL is detected by combining clinical symptoms with microscopic identification of the pathogen in Giemsa stained (nucleic-acid stain) skin scrapings or needle aspirates. Sample collection is often an invasive procedure and both diagnostic methods require medical expertise and are labour intensive. Furthermore, microscopic examination generally often has low sensitivity. Thus, nucleic acid amplification tests (NAATs), such as PCR or nucleic acid sequence-based amplification (NASBA), which have increased sensitivity when compared to microscopy, have been developed. However, these nucleic-acid-based methods are challenging to implement in resource-limited endemic regions due to high costs and lack of resources.[32]

PoC test
There are three PoC tests for the diagnosis of CL. CL Detect™ Rapid Test is an immunological PoC test that detects the *Leishmania* antigens in ulcerative skin lesions. However, this test lacks specificity which results in a high number of false positive test results. The Loopamp™ *Leishmania* Detection Kit (loopamp) is an isothermal loop-mediated NAAT that amplifies the conserved region in the 18S rRNA of *Leishmania* and a specific sequence of the kinetoplast DNA of *Leishmania donovani*. The Loopamp is not a true PoC, as it requires DNA extraction prior to testing, but its relative simplicity makes it a possibility as a 'near'-PoC diagnostics test.[32] The palmPCR, which is a hand-held battery-operated device that targets the conserved region in the kinetoplast DNA of *Leishmania*, works very well, but it requires a trained clinician.[33-36]

Implementation need

While there are already several PoC devices for CL available with moderately good specificity and sensitivity, these tests need to be validated in the field. Field-deployable sample preparation is required to detect early CL infections in resource-limited settings.

2.6.7 Mycetoma

Mycetoma is a chronic, debilitating, and socially stigmatizing skin disease that is caused by multiple microorganisms, bacteria (actinomycotic) and fungi (eumycotic) which reside in soil. Actinomycotic bacterial species such as *Streptomyces somaliensis, Actinomadura madurae, Actinomadura pelletieri, Nocardia brasiliensis* and *Nocardia asteroides,* and eumycotic fungal species like *Madurella mycetomatis, Madurella grisea, Pseudoallescheria boydi*i and *Leptosphaeria senegalensis* are common causal agents for mycetoma. The infection affects the feet, upper extremities, and back. Though there are no reports on human-to-human transmission or animal reservoirs (notably, it also infects animals), the disease remains endemic in certain regions due to shared environmental factors and occupations such as farming.[37] Mycetoma is endemic in many tropical and sub-tropical countries constituting a 'mycetoma belt'. Mycetoma is also associated with poor personal hygiene, unavailability of protective clothing and shoes, and weaker immunity of hosts. Although mycetoma is uncommon among females, more active and aggressive forms of this disease have been observed during pregnancy possibly due to hormonal effects and suppressed immune response during pregnancy.[38] Approximately 840 cases were reported in 2016, although this number likely underestimates the burden of this disease.[2] Available antifungal drugs are generally ineffective, toxic, expensive, and unavailable in endemic regions, and anti-bacterial therapies require uninterrupted administration to avoid the development of resistance. Treatment is more successful in actinomycetoma (bacterial) than eumycetoma (fungal), and requires a holistic approach comprising antimicrobials, surgery, and rehabilitation.[39] Clinical presentation of both actinomycotic and eumycotic mycetoma is identical and their differentiation is therefore required for proper case management. The time from the onset of the initial infection to seeking healthcare ranges from 3 months to 50 years, and a diagnostic delay can amount to 15 years due to symptoms that are similar to other prevalent diseases which causes misdiagnoses.[40] Thus, case management requires a detailed understanding of other infectious diseases that mimic mycetoma, including elephantiasis, yaws, chronic bacterial osteomyelitis, and other fungal diseases, and non-infectious diseases such as tumors [38].

Current diagnostics

Clinical diagnosis requires equipped facilities and trained personnel. Imaging procedures including ultrasound, radiography, CT and MRI scans are needed. Identification of the causal agent is also crucial to guide the treatment procedure and

therefore, fine needle aspiration for extraction of mycetoma grains for microscopic staining and histopathology are also employed.[40] Molecular diagnostics using 16S rRNA and sequencing, PCR, and LAMP are new methods that are being developed.[37] Immunological tests have also been developed, including immunoblots and ELISA.[40] Existing diagnostic methods are inadequate and cannot be used as PoC tests in resource-limited settings.

PoC test

There are currently no commercial PoC tests for Mycetoma.[41]

Implementation need

Sensitive and species-specific immunological tests or nucleic-acid-based PoC tests with the possibility to function as test-of-cure are required for case detection and to monitor therapeutic outcomes, respectively.

2.6.8 Human African Trypanosomiasis (Rhodesiense)

Human african trypanosomiasis (HAT) (sleeping sickness), is a parasitic protozoan disease caused by two species from the genus *Trypanosoma*: *Trypanosoma brucei rhodesiense* (*T.b.rhodesiense*, rHAT, 2% cases) and *Trypanosoma brucei gambiense* (*T.b. gambiense*, gHAT, 98% cases; described later). Infection occurs after a bite from the tsetse fly that has fed off other infected individuals. Infection could also be congenital (mother-to-child), through other bloodsucking vectors, accidental infection in a laboratory or sexual transmission, although these are less common than via tsetse flies. HAT has two stages: the haemolymphatic and neurological (meningoencephalic) stage. First, the parasite moves to the subcutaneous tissue and the blood and lymph vessels where it starts multiplying causing fever, headache and enlarged lymph nodes. Later, the parasites cross the blood-brain barrier and infect the central nervous system, where they cause a change in behaviour, confusion, sensory disturbances, and disturbance of the sleep cycle. HAT is fatal if left untreated. Globally, ~ 51 million individuals are at risk for gHAT and ~3 million individuals are at risk for rHAT, mostly in resource-limited settings in sub-Saharan Africa.[43,44] rHAT is treated with suramin during the haemolymphatic stage and melarsoprol during the neurological stage.[2]

Current diagnostics

Microscopy for parasite detection is a valuable diagnostic method for rHAT as the acute phase corresponds with high parasitaemia levels (blood, lymph nodes or cerebrospinal fluid). Capillary tube centrifugation is an effective sample preparation techniques. Staining of the parasite with UV fluorescent dye can also be applied to diagnosing rHAT. A lumbar puncture is used to stage rHAT i.e. to determine if the parasite has reached the central nervous system. Nucleic acid-based approaches for rHAT include a technique called OC-PCR, wherein PCR is coupled to oligochro-

matography, which has been developed as a proof-of-principle study. LAMP and NASBA show great potential, as it has very high specificity. However, these nucleic-acid-based approaches are not commercially available.[45-47]

PoC test

There are currently no commercial PoC tests for rHAT.

Implementation need

The identification of novel circulating biomarkers for rHAT is required to ensure the timely diagnosis of this disease in endemic resource-limited settings. A field-deployable nucleic-acid-based test would facilitate the species-specific detection of rHAT, which will aid effective treatment.

2.6.9 Buruli ulcer

Buruli ulcer is a disabling and stigmatizing disease that is caused by *Mycobacterium ulcerans* (*M. ulcerans*). It is the third most common mycobacterial disease after tuberculosis and leprosy. The mode of bacterial transmission is currently unknown. Infection with *M. ulcerans* causes ulcers that affect the skin, soft tissues and bone, resulting in a chronic debilitating illness that persists throughout the infected persons life-time. The initial symptoms are painless dermal papillae or sub-cutaneous nodule that are common to many skin NTDs, and thus diagnoses are often delayed, resulting in deteriorating patient conditions. Buruli ulcer is endemic across the global south affecting as many as 33 countries in Africa, Latin America, Australia and Japan.[2] Despite the availability of effective treatment i.e., a prolonged course of a combination of antibiotics (typically rifampin and clarithromycin injections), ~2700 new cases were reported in 2018. Ref[2,48] Once the disease has progressed, treatment relies exclusively on surgery to remove the affected regions, including excision, skin grafting and possibly limb amputations. Disease management is difficult with HIV co-infection and other co-existing endemic NTDs in the tropical regions.[49]

Current diagnostics

Current diagnostic practices are conducted at the primary healthcare level or one level up, and thus require trained personnel and sophisticated instrumentation. Conventional methods for diagnoses include empirical clinical methods and microscopy. Other methods including histopathology, bacterial culture methods, enzyme linked immunosorbent ELISA assays, and molecular diagnostics such as PCR (targeting the IS_{2404} sequence) and fluorescent thin-layer chromatography (f-TLC) are also performed based on availability. In 2018, along with the WHO, the Foundation for Innovative New Diagnostics (FIND) started a joint-project for the evaluation and implementation of f-TLC to detect the unique toxin mycolactone in lesions using monoclonal antibodies.[50-52]

PoC test

FIND is developing and piloting PoC tests for Buruli ulcer in endemic regions. The tests range from serological tests i.e., an immunoassay targeting MUL_3720 protein, to NAATs based on LAMP and recombinase polymerase amplification (RPA).[52] The detection of 16s rRNA as a target is also possible. However, these tests may not be easily administered as a PoC test as they require a basic laboratory for sample preparation (DNA extraction).[50] A non-instrumented nucleic acid-based device is under development for providing an electricity-free incubation that utilizes a LAMP based test for the detection of *M. ulcerans* DNA, which may be suitable for field applications upon further development.[53]

Implementation need

There is a need for a field-deployable PoC test that can detect viable bacterium (active infection).

2.6.10 Schistosomiasis

Schistosomiasis is caused by an infection with trematode flatworms (schistosomes). *Schistosoma (S) haematobium, S. mansoni* and *S. japonicum* are the most common infective subspecies that cause human schistosomiasis. Schistosomes spread through fresh water that is infected with trematode larvae. After infection, the larvae settle in the liver where they mature into worms. Adult worms lay eggs in the blood vessels of the patient, and these eggs secrete antigenic glycoproteins which facilitate the passage of the eggs from the blood to into the internal organs or urinary bladder, where they induce an inflammatory response.[54] The eggs of adult schistosomes leave the human body in urine or faeces and hatch in fresh water where they penetrate snail hosts. In these hosts, the larvae develop and multiply cercariae (free-swimming infectious stage of schistosomes) that emerge from the snails and can penetrate human skin.[55] Symptoms of acute schistosomiasis are myalgia, diarrhoea, fatigue, malaise, and fever. Active infections cause inflammatory reactions in tissues of the host. Schistosomiasis is widespread in the tropics and subtropics, especially in poor communities without clean water and adequate sanitation.[56] ~700 million people are at risk of acquiring the infection as they live in endemic areas. 143 million people worldwide were infected in 2017, and approximately 2.5 million DALYs were estimated in 2016.ref[54,57] Schistosomiasis is treated with praziquantel which is effective for all schistosome species.[2]

Current diagnosis

S. haematobium eggs are excreted in urine and can be detected in can be detected by observing the sample under a microscope.[58] To increase the sensitivity, urine samples are often concentrated via filtration, sedimentation or centrifugation. In general, microscopy lacks sensitivity for all species as egg excretion is variable during the disease. The prevalence and intensity of infection with *S. mansoni* and *S.*

japonicum is tested the Kato-Katz technique that relies on microscopic analysis of stool samples.[135] This method is simple and inexpensive. However, it lacks sensitivity and cannot detect early infections.[55] Serological detection of anti-schistosome antibodies is also used. However, the specificity of the method is sub-optimal, and serological tests cannot distinguish between *Schistosoma* species or between active and inactive schistosomiasis.[58] In-high resource settings, NAATs such as PCR can be used to detect the disease in serum samples to distinguish between different Schistosoma strains and are highly specific.[59] In endemic settings, PCR on stool or urine is occasionally used for epidemiological studies (certainly not for routine diagnostics).

PoC test

A PoC-CCA test detects circulating cathodic antigen (CCA) in serum or urine[60]. This specific assay can detect *S. japonicum* and *S. mansoni* but not *S. haematobium*.[61,62] A rapid immunochromatography test (*Schistosoma* ICT IgG-IgM) is commercially available.[63] A smart diagnostic device known as the schistoscope is also under development.[64] A field deployable upconverting particle lateral flow (UCP-LF) assay for the detection of circulating anodic antigen (CAA) in serum or urine is currently under development.[65]

Implementation need

The UCP-LF CAA test is currently undergoing field studies. This antigen-based test or a new nucleic-acid-based PoC test would contribute to specific and early detection. Detection of *S. haematobium* infection for mapping in low prevalence areas is also needed.

2.6.11 Chagas disease

Chagas disease (CD, also known as American trypanosomiasis) is a vector-borne disease that is caused by the parasitic protozoan, *Trypanosoma cruzi*, after the bite of an infected blood-sucking triatomine bug. *T. cruzi* is spread through mucous membranes (eyes, mouth) and breaks in the skin (such as a wound). The dominant vectors for human transmission stem from the genera *Triatoma, Rhodnius* and *Panstrongylus*. Although CD is mainly a vector-borne disease, other forms of transmission such as congenital transmission, through blood transfusion, organ transplantation, and the consumption of contaminated uncooked food have caused the disease to spread worldwide.[2] During the acute phase of the disease, patients are either asymptomatic or develop mild generic symptoms such as a fever, headache, a rash, or vomiting. Approximately 90 days after infection, patients become asymptomatic and this marks the chronic phase, during which ~30% of the patients develop symptoms, such as cardiac or gastrointestinal complications. Without treatment, the chronic phase can last from decades to an entire lifetime, while chronic cardiomyopathy can be fatal. Globally, CD is estimated to affect ~8 million people and is responsible for 219,000 DALYs.[42,66,67] CD is treated with the antiparasitic drugs

nifurtimox and benznidazole.

Current diagnostics

During the acute phase, microscopic examination of a blood smear can be used to identify CD due to high parasitaemia. During the chronic phase, parasitaemia decreases and microscopy results cannot be used to confirm a CD diagnosis. Serological tests to detect *T. cruzi* antibodies are often used to diagnose CD. Serological tests indirect immunofluorescence assays and ELISA. Detection of parasitic DNA through PCR amplification of the minicircle fragment of the kinetoplast DNA and a 188 bp satellite sequence of nuclear *T. cruzi* DNA can also be used to detect CD. However, all these diagnostic tests require laboratory expertise and are either expensive or labour intensive.[68,69]

PoC test

There are multiple PoC immunochromatographic tests that are commercially available. However, only a number of these show sufficient specificity and sensitivity and are easy enough to use, but confirmation of test results in a laboratory is recommended in practice.[70] Single-use immunochromatographic tests such as *Trypanasoma* DetectTM are commercially available for the detection of antibodies against *T. cruzi.* There are proof-of-principle studies on PoC tests, such as the serological PATH-Lemos rapid assay and TESA (Trypanosomal Excreted/Secreted Antigens lateral flow test). However, these proof-of-principle tests are not commercially available yet. [71,72]

Implementation need

A nucleic-acid-based PoC test to detect congenital CD PoC test is urgently needed.[2]

2.6.12 Visceral leishmaniasis

Visceral leishmaniasis (VL, also called kala-azar (black fever)), is a fatal form of leishmaniasis (described previously for cutaneous leishmaniasis) if left untreated. VL is predominantly a result of infection with *L. donovani* and *L.infantum*. Symptoms include fever, weight loss, an enlarged spleen and liver, and severe anaemia. VL does not have a specific endemic area, but 95% of all new cases are in Brazil, China, Ethiopia, India, Iraq, Kenya, Nepal, Somalia, South Sudan and Sudan. After treatment, VL can develop into Post-kala-azar dermal leishmaniasis (PKDL), which becomes a reservoir for the parasites. PKDL usually occurs 6 months to several years after a VL patient is treated.[73,74] VL can treated with liposomal amphotericin B (AmBisome®), which is administered by intravenous infusion.[75] However, due to high costs and/or unavailability of AmBisome®, miltefosine or pentostam can be used to treat VL.

Current diagnostics

VL can be diagnosed by microscopic confirmation of the parasite in bone marrow, spleen or lymph node aspirates. This requires medical expertise and often involves an invasive procedure.[76] The direct agglutination test (DAT) is widely used, which detects the presence of antibodies against the *Leishmania* parasite. However, a trained technician is required to administer the test and results are subjectively interpreted which leads to inter-reader variation.[77-79]

PoC test

The IT-LEISH® Kit is a prominent immunological test that detects antibodies against the rK39 antigen.[80] This test has been widely used in India, but it fails to reliably detect VL in Africa. Other immunological tests have also been developed to detect the rK28 antigen.[81] However, these tests fail to distinguish between current and past infections.[82-84] Another PoC test is the duplex visceral leishmaniasis lateral flow device (VL-LFD) based on a laser-patterned microfluidic assay that detects two recombinant *Leishmania* antigens: *β-tubulin* and *LiHyp1*. However, this test is not commercially available.[85] Also, the *Leishmania* Ab Rapid Test and the Kalazar Detect™ Rapid Test are commercially available, but these two tests are only able to detect *L. donovani* infections and should therefore always be combined with additional testing.[86] There is a mobile suitcase that has been developed for PoC diagnostics,[87] however it is not routinely used in endemic settings.

Implementation need

The rK39 rapid immunochromatographic test is not effective in east Africa. Field validation of the mobile suitcase and VL-LFD are required.[87] A field-deployable nucleic-acid-based test (test-of-cure) is crucial for the diagnosis of post-kala-azar (PKDL), and it would improve the prompt diagnosis of VL in resource-limited endemic regions.[88]

2.6.13 Lymphatic filariasis

Lymphatic filariasis (LF, also known as elephantiasis) is caused by an infection with one of three nematode species (parasitic worms) from the family *Filarioidea*. Infection with *Wuchereria bancrofti* accounts for 90% of the infections, and *Brugia malayi* and *Brugia timori* accounts for the remaining 10%. The parasites are transmitted to humans by various mosquito species. LF affects the lymphatic vessels and immune system of the patient. When infected with the parasite, the worms settle in dilated nests within the lymphatic vessels where they produce millions of microfilaria (larvae) that migrate to the bloodstream. The parasite can then enter an intermediate host, the mosquito, which is the start of a new cycle of infection.[89] Asymptomatic patients still get lymphatic and kidney damage. This disease is com-

monly found in South-East Asia and Africa. More than 40 million people have the clinical manifestations such as lymphoedema of the limbs (elephantiasis).[89] Globally, approximately 50 million people were infected with LF in 2017, 890 million people living in endemic areas require MDA, and ~1,2 million DALYs were estimated in 2016.[2] Diethylcarbamazine treatment is effective against LF, however, DEC cannot be used in regions where onchocerciasis and loiasis are present. LF can be treated with MDA of ivermectin and albendazole, especially in settings where onchocerciasis is present.[90]

Current diagnostics

The reference standard diagnosis of LF relies on the detection of microfilariae in blood samples by microscopic examination.[91,92] Routine laboratory assays such as ELISA can also be used to detect elevated levels of antifilarial IgG4 antibodies.

PoC test

There are multiple PoC tests for lymphatic filariasis that are commercially available. The Alere™ Filariasis Test Strip detects antigens of *W. bancrofti*. Other commercial tests include rapid anti-filarial antigen-based tests such as *Brugia* Rapid™ and SD BIOLINE Lymphatic filariasis.[92,93]

Implementation need

Current PoC tests that detect antigens of *W. bancrofti* have cross-reactivity with antigens of the infectious disease loiasis (African eye worm), which has been recently recognized as a serious obstacle to eliminate LF in loiasis-endemic areas.[93] A PoC test without cross-reactivity with loiasis could contribute to the elimination of LF. Novel biomarker identification is also required to facilitate the development of multiplexed tests for LF and loiasis.

2.6.14 Chikungunya

Chikungunya is a viral disease that is caused by an alphavirus in the family *Togaviridae*, which is transmitted by the bite of female mosquitos *Aedes aegypti* and *Aedes albopictus*. This disease shows similar transmission and symptomatic patterns as dengue and malaria infections. Chikungunya can also exhibit latrogenic transmission via blood transfusions or organ transfusions (liver, kidney etc.). Since the chikungunya virus has been detected in semen 30 days after symptoms onset, sexual transmission is also a possibility, but no evidence for this has been reported so far. Chikungunya is an emerging seasonal febrile illness that is characterized by intense joint pain and it can cause lifelong effects such as arthritis, chronic inflammatory rheumatism, and neurological manifestations.[94] The exact burden of this disease is unknown, however, the WHO reports that there have been approximately 2 million infections since 2004 disease.[2] Disease management is difficult during pregnancy as it can impact fetal development, and cause neonatal infections resulting in severe

acute cases of microcephaly and cerebral palsy. With an unprecedented increase in chikungunya spread across more than 60 countries, there is an urgent need to combat this disease. Although vaccination programs have been initiated, eradication is still dependent on early diagnoses for effective treatment. There is no specific treatment for chikungunya, but the symptoms can be alleviated with medication.[96]

Current diagnostics

Laboratory based tests are used to diagnose chikungunya in serum or plasma to detect viral nucleic acids or virus-specific IgM at 5 to 7 days after symptom onset. Specific chikungunya virus IgG can be detected post treatment for several years. There is a possibility of false positive test results due to cross-reactions with other alphaviruses. RT-PCR is used to detect the chikungunya virus and is performed 7 days after the onset of symptoms. Viral cultures in biosafety level 3 laboratories are also used to detect chikungunya. The clinical challenge lies in the inability to distinguish chikungunya from dengue as both diseases usually occur during the same seasons. Serological tests, including ELISA, can confirm the presence of antibodies. While RT-PCR methods are available to detect the virus in blood samples, their sensitivity varies and they are not suitable for use in endemic resource-limited settings.[94]

PoC test

An antibody screening test for chikungunya has been developed that detects IgM antibodies in a solid-phase immunochromatographic assay (SD BIOLINE Chikungunya), and there is the Chikungunya IgM Combo Rapid Test (CE) that is commercially available. A proof-of-principle study for chikungunya virus genome utilizing the combination of a modified reverse transcription loop-mediated isothermal amplification (RT- LAMP) method and a MinION sequencer is being developed.[97]

Implementation need

There is an urgent need for a field-deployable PoC test for chikungunya that does not have any cross reactivity with dengue. A multiplexed test with dengue would also greatly improve the diagnosis and treatment of chikungunya. Novel biomarker identification would facilitate the development of a multiplexed text that does not have any cross reactivity with dengue.[98]

2.6.15 Scabies

Scabies is a curable skin infestation that is caused by microscopic mites known as *Sarcoptes scabiei var. hominis*.[99,100] Scabies is highly contagious, and is transmitted from person-to-person through close skin contact. Once infected, the female mite settles in the skin and lays eggs, thus triggering an immune response that causes intense itching and a rash. The skin is then exposed to bacterial infections that can cause severe soft-tissue infections, septicaemia, kidney disease, and rheumatic fever. Approximately 455 million people are infected by scabies at any time, causing

~5,6 million DALYs (2016).ref[101] Although people of all ages can become infected, scabies mostly affects children. Outbreaks in residential and nursing homes for the elderly are common in high-resource settings.[100] Immunocompromised people, such as HIV positive people, are more prone to develop crusted scabies.[99] MDA using oral ivermectin and topical scabicides is an effective treatment strategy. Topical scabicides include permethrin, benzyl benzoate, malathion, and sulphur ointment.

Current diagnostics

Scabies is diagnosed empirically, using the patient's history and a physical examination. Scabies presents with visible symptoms within 2-6 weeks of the initial infestation. Secondary bacterial infections with *Staphylococcus* or *Streptococcus*, as a result of scratching, are often misleading. Definitive diagnosis relies on microscopy to identify mites or eggs from skin scrapings. However, treatment is often started without microscopic confirmation. Serological methods have not been successful in detecting human infections.[102] DNA libraries of *S. scabiei var. hominis* have been constructed for epidemiological studies, but commercial molecular diagnostic tests have not been developed.[102] Essentially, there is no standard diagnostic procedure which leads to misdiagnoses.[100,103] Critical action is required to improve individual diagnosis as misdiagnosis and delayed treatment are the main challenges to eradicating scabies.[104]

PoC test

There are two PoC tests for scabies, the burrow ink test and the handheld dermatoscopy microscopy tool.[102] The burrow ink test is a simple non-invasive test than can be used to screen a large number of patients. A fountain pen is gently rubbed onto a suspected lesion. Excess ink is wiped off with an alcohol swab, making the infected site (burrow) visible with a wavy ink-filled line where the mites have tunnelled into the skin. The handheld dermatoscopy tool is accurate but it is expensive and requires trained users.[105]

Implementation need

There is a need for diagnostic methods that are standardized and can take place at the PoC. This would be beneficial in reducing scabies, predominantly by preventing misdiagnosis and delayed treatment.[100]

2.6.16 Onchocerciasis

Onchocerciasis (river blindness), caused by a parasitic worm *Onchocerca volvulus,* is a one of the NTDs that is slowly approaching elimination.[106] It is an eye and skin disease that is predominantly found among inhabitants living nearby rivers that breed *Simulium* black flies, which transfer adult female worms to a human body during a blood meal. A single adult female worm produces thousands of microfilariae (larvae) which migrate into the skin and eyes. The death of these microfilariae

results in the release of toxic chemicals into the eyes and skin, eventually causing blindness and disfigured skin.[107] Onchocerciasis has been endemic within Africa and the Americas with approximately 21 million cases in 2017 and approximately 205 million DALYs in 2017.ref[108] MDA of Ivermectin and vector control strategies facilitated the containment of the disease, bringing it now close to the elimination of transmission.[109]

Current diagnostics

While microscopic examination of skin biopsies is the reference standard for the diagnosis of onchocerciasis, molecular diagnostics using PCR can be performed as a test-of-cure. Real-time PCR and LAMP assays can also be performed, as they can provide a rapid colorimetric readout to detect even a single parasite.[110] Immunological diagnoses using ELISA have been the most economical diagnostic tests, though they still require cold chain, equipped laboratory, and trained personnel, thus making it unsuitable for diagnosis in endemic resource-limited settings.

PoC test

There is a SD BIOLINE biplex PoC test for *Onchocerca volvulus* and *Wuchereria bancrofti* antigens, and a SD BIOLINE Onchocerciasis IgG4 test that detects antibodies in a sample in response to Ov16 antigens.[111]

Implementation need

Current PoC tests that detect *W. bancrofti* antigens have cross-reactivity with antigens of the infectious disease loiasis. A field-deployable PoC test that does not have cross reactivity with *Loa Loa* antigens is required, as co-infection with onchocerciasis and loiasis affects the treatment regimen. Novel circulating biomarker identification is required to identify specific biomarkers that do not cross react. A confirmatory test-of-cure PoC would facilitate the eradication of this disease, and surveillance thereafter.

2.6.17 Human African trypanosomiasis (gambiense)

HAT caused by *T.b. gambiense* (gHAT) accounts for 98% of all reported cases and occurs in 24 countries primarily in western sub-Saharan Africa. Similar to rHAT (described above in the page for rHAT), gHAT also spreads through the bite from a tsetse fly (vector). gHAT involves a long haemolymphatic stage, and it often remains undiagnosed due to the generic symptoms. After several months or even years, gHAT progresses to the neurological stage. Parasitaemia remain very low throughout both stages, which makes it difficult to diagnose gHAT from body fluid samples.[2,44] Treatment for gHAT includes pentamidine (haemolymphatic stage); eflornithine and nifurtimox (neurological stage).[2,112]

Current diagnostics

Diagnosis of gHAT relies on the detection of antibodies using immunological tests (screening), and microscopy for parasite detection and stage diagnosis to determine the type of treatment that should be administered.[47] Due to low parasitaemia levels of *T.b. gambiense,* detection mostly depends on immunological screening. The most common immunological test is the card-agglutination test for trypanosomiasis (CATT), which is an antibody detection test that binds to the LiTat 1.3 antigen (surface glycoprotein) present in *T. b. gambiense.* However, this screening test must be followed by microscopy.[113] Techniques that are based on staining the parasites with fluorescent dyes and UV visualisation can also be applied to diagnose gHAT. The OC-PCR technique, LAMP and NASBA tests that were decribed previously for rHAT can also be used for gHAT. However, these nucleic-acid-based tests are not commercially available.[45-47]

PoC test

SD BIOLINE HAT (developed by FIND and Standard Diagnostics is a serological PoC lateral flow test for gHAT. SD BIOLINE HAT detects anitbodies that bind to LiTat 1.5 and LiTat 1.3 antigens. This test was deployed in endemic regions in 2014.[113] In 2018, a new version of this PoC test was introduced (SD BIOLINE HAT 2.0) using recombinant antigens.[114] A rapid serodiagnostic PoC test called HAT Sero-*K*-SeT is also based on LiTat 1.5 antigens, and showed a comparable sensitivity and specificity to the CATT.[115] Other lateral flow assays based on multiple antigens have been reported to detect gHAT, but these tests are not commercially available yet.[116]

Implementation need

gHAT needs to be diagnosed early to avoid progression into the neurological stage, for which only complicated, risky, and expensive diagnosis and treatment are available. Screening of the population that is at risk is key to diagnose gHAT as early as possible. Therefore, a cost effective and easy to use PoC test is required with higher sensitivity and specificity than current immunological based PoC tests to do quick screenings of whole populations. FIND is actively developing and evaluating such tests. Test-of-cure tests are also needed to replace the cumbersome and invasive lumbar puncture, and to guage efficacy of treatment.

2.6.18 Snakebite envenoming

Snakebite envenoming (SBE) is caused by the injection of venom into the patient after the bite of a venomous snake, while certain snake species spray venom directly into the patient's eyes.[117] Snakebites can be fatal as the toxins can cause paralysis that prevent breathing, or bleeding disorders that can lead to fatal haemorrhaging.[118] Other SBE effects include irreversible kidney failure and tissue damage that can lead to permanent disability or limb amputation. Approximately 2,7 million

people are bitten by snakes with envenoming annually, causing 80,000 – 140,000 deaths, and ~6-8 million DALYs in 2015.ref[2] The availability of antivenom treatment (venom neutralizing antibodies) is expensive and limited in supply, and has a number of associated adverse effects, making administering antivenom risky.[119] Antivenom cannot reverse tissue damage caused, but it can neutralize active circulating venom to prevent further tissue damage.[117] A reliable diagnostic tool would alleviate the damage caused by SBE, but it is unlikely that snakebites in general can be eradicated.

Current diagnostics

Empirical diagnosis is often used to diagnose SBE. Clinical symptoms include blistering, swelling, bleeding, tissue necrosis and pain. Verbal confirmation from the patient is also used to verify SBE if the patient is lucid. When symptoms are less clear, a 20-minute whole blood clotting test is used to determine SBE. In such tests, it is essential that the glassware is standardised, and the sample volume and temperature need to be regulated and validated using serial donor samples prior to routine use. More specific molecular tests are being developed but are not considered PoC tests as they rely on laboratory-based blood coagulation analysers.[119]

PoC test

There is one commercial antibody-based diagnostic test, the Australian commonwealth serums lab snake venom detection kit.[2] The major limitation of this test is that it is a matching tool for 5 of the Australian antivenom treatments. There is also cross reactivity between venoms, which leads to false positive results.[119]

Implementation need

A PoC test is needed to detect venom-induced coagulopathy, a bleeding disorder that occurs when the bloods ability to coagulate is impaired.[119] Species specific diagnosis is not essential for effective treatment[2] as polyvalent antivenom (against different species) is produced more commonly than monovalent antivenom (against a single species). A more practical need is a region-specific multiplexed PoC test, as snake species are geographically separated.[120] The international normalized ratio (INR) is a screening method for venom-induced consumption coagulopathy after snakebites. INR currently relies on a laboratory coagulation analyser, and studies have demonstrated that the PoC INR is not reliable as it results in false negative test results.[121] Further research is required to develop a reliable PoC coagulation analyzer which would enable more reliable PoC diagnosis of venom-induced coagulopathy.

2.6.19 Dengue

Analogous to chikungunya, dengue is also an acute arboviral disease that is transmitted by the bite of an infected female *Aedes* mosquito. Non-vector transmissions through blood transfusions, organ transplantations, mucosal splashes have also been reported. Although the virus has been found in semen and vaginal secretions, no confirmed cases of sexual transmission have been reported. While vertical transmission from a viraemic mother to her foetus through placental transmission is plausible, increasing the risk of preterm birth or fetal death, transmission to infants though breast feeding has been ruled out. Dengue infections occurs over a period of 3-4 weeks until recovery. This disease presents in different phases ranging from a febrile phase with a headache, malaise and general body pain from the onset of the disease, to the critical phase wherein plasma leakage can lead to the respiratory distress, persistent vomiting, abdominal pain, and secondary infections. The pathogenicity in severe cases is due to cross-reactivity of antibodies (antibody-dependent enhancement), resulting in the greater burden of the infection to the vascular system which leads to dengue shock syndrome,[122] coagulation abnormalities, and bleeding in hepatic and neurological organs, and also renal and ocular impairment. Dengue occurs globally and results in a socioeconomic and disease burden on the healthcare facilities in tropical and sub-tropical regions. There are approximately 400 million infections per year[123] and 3 million DALYs globally. Due to international travelling of asymptomatic carriers and patients exhibiting minor symptoms, dengue has shown a rapid geographic spread and cases have been reported in temperate climates such as in USA and Europe (Croatia, France, Portugal).[2] Recent research has focused on vaccine development and transmission control measures.[122] There is no specific treatment for dengue, but the symptoms can be alleviated with medication.[124]

Current diagnostics

There are multiple laboratory-based diagnostic methods for the detection of the dengue virus, including virus isolation in cell culture, viral RNA detection by NAATs such as RT-PCR, and the detection of viral antigens such as the NS1 antigen detection test that detects secreted proteins in blood samples. ELISA can be performed 4-5 days after the onset of initial symptoms. Since dengue IgM antibodies start to increase from day 4 onwards, peak at about days 10–14, and then decline and disappear after about 3 months, serological assays are preferred. Cross-reactivity with Zika virus is reported for all immunological assays as well as the NS1 antigen test.[122]

PoC test

There are multiple commercial immunological PoC tests, such as the SD BIOLINE Dengue Duo (Dengue NS1 AG+IgG/IgM) which is an antibody-based (to detect IgG and IgM) and antibody-based (to detect NS1) test, and the ASSURE®Dengue IgA Rapid Test (antigen-based). [125-127]

Implementation need

Newly developed tests will need to be validated in the field in endemic regions. A field-deployable PoC test that does not have any cross reactivity with the Zika virus is required. Novel circulating biomarker identification is required to identify specific biomarkers that do not cross react. A multiplexed test with the Zika virus can then be developed.[104]

2.6.20 Dracunculiasis

Dracunculiasis (Guinea-worm disease) is a disabling parasitic disease that is caused by the nematode Guinea worm *Dracunculus medinensis*. It is transmitted to people from drinking stagnant water with parasite-infected water fleas that contain the larvae of *D. medinensis.* Infected patients often immerse their limbs into water to relieve the burning sensation when the worm is emerging. The worm then releases larvae into the water, completing the circle of transmission. Animals are also infected, especially dogs. Although this disease is rarely fatal, patients become non-functional for weeks when the adult female worms emerge from the body. Surgical removal of the worm is possible in high-resource settings.[2,128] Dracunculiasis is on the verge of eradication with only 28 human cases reported in 2018 and 54 cases reported in 2019.[2,104] In terms of eradication, only 7 countries remaining need to be certified as free of the disease (Angola, Chad, Ethiopia, Mali, South Sudan, Sudan, and the Democratic Republic of Congo). There is currently no treatment or vaccine to prevent this disease.[2,104] Current eradication strategies, which proved effective, involved treating bodies of water with temephos (larvicide) to provide safe drinking water, and providing health education to increase awareness in affected communities.

Current diagnostics

Dracunculiasis is diagnosed by clinical presentation, based on the appearance of a skin lesion with a protruding worm that can be seen with the naked eye, usually on the lower limb but also on the abdominal region. Blood tests reveal elevated eosinophilia and immunoglobulin G4 levels. Alternatively, x-rays can reveal dead calcified worms if the worms die before they exit the skin. Immunological diagnostic tests are not useful in practice as it has not been determined if they can detect prepatent infections (the period between infection with a parasite and the demonstration of the parasite in the body) – mainly due a lack of prepatent serum samples.[129] Evidence has not been found for circulating antigens during prepatent infections. There is evidence for antibody detection during patent infections against whole-worm antigens.[129]

PoC test

There are currently no commercial PoC tests for dracunculiasis.

Implementation need

Testing bodies of water are more useful than testing individuals as the patient symptoms are very clear. Surveillance is the key to ensure eradication certification to countries that are on the verge of eradication. There is a need for a PoC test for humans to aid ongoing surveillance, and a need for a pond-side test for detecting *D. medinensis* DNA in copepods.[2]

2.6.21 Leprosy

Leprosy is a chronic infectious disease that is caused by the bacteria *Mycobacterium leprae.* Known routes of transmission of *M. Leprae* are droplet infection via the nose and mouth, or via skin-to-skin contact. When infected, *M. leprae* patients enter the relatively asymptomatic early disease stage, and it can take 5-10 years before clinical symptoms arise. These symptoms are characterized by hypopigmented macules, which are common characteristics of other skin diseases and which rarely leads to the diagnosis of leprosy.[130,131] If left untreated, the disease may develop into a permanent stage of leprosy. The WHO classifies permanent leprosy in two clinical variants: paucibacillary leprosy where the immune system of the patient is sufficient, and multibacillary leprosy where the immune system of the patient is poor. This classification is useful for diagnosis and treatment in the field. The permanent stage is classified by skin lesions, damage to the nervous system, and eventually disfigured mutilations of limbs.[131] The highest incidence of patients are found in India, Brazil, and Indonesia.[132] The exact burden of this disease is unknown.[2] Leprosy is commonly treated with a single dose of rifampicin to all contacts of positive cases, and mutli-drug therapy lasting 6-12 months with a combination of dapsone, rifampicin, and clofazimine.[131]

Current diagnostics

Diagnosis of leprosy is difficult as it does not have unique characteristics. Leprosy is clinically diagnosed if patients show two out of three typical signs, defined as loss of sensation in a skin lesion, enlarged peripheral nerve, and positive detection of bacilli in skin smears from the lesion. Laboratory-based tests have difficulties in detecting certain forms of leprosy, and thus clinical diagnosis is still necessary.[130] Microscopic detection of *M. leprae* infection is done by observing stained bacteria in lymph fluid or skin lesions.[131] ELISA and lateral flow assays have low accuracy. Therefore, the most sensitive laboratory diagnostic tool for *M. leprae* is PCR which targets *M. leprae* specific genes.[130,131] Where the sensitivity and specificity of PCR tests is close to 100% for multibacillary leprosy, PCR only has sub-optimal sensitivity and specificity for paucibacillary leprosy.[133]

PoC test

There are currently no PoC tests that are commercially available for the diagnosis of leprosy. However, a recent study showed promising results for a lateral flow assay

test that is based on specific biomarkers for leprosy (ApoA1, IL-1Ra, S100A12).[134]

Implementation need

Newly developed tests, such as the lateral flow assay discussed above, will need to be validated in the field before it becomes commercially available. Diagnosis of infection and/or screening for potential infections is needed to control disease transmission across populations.

2.6.22 Soil-transmitted helminthiases

Soil-transmitted helminthiases (STHs) are a group of intestinal parasites that include *Ascaris lumbricoides, Strongyloides stercoralis, Trichuris trichiura,* and hookworms *Necator americanus* and *Ancylostoma duodenale*. STHs are transmitted, depending on the species, either by eggs or larvae present in human faeces, and are highly prevalent in many tropical areas with poor sanitation. STHs cause aneamia, malnutrition, impaired growth and cognitive development, abdominal pain, diarrhea, and hyperinfection syndrome. There were 1.5 billion people infected with STHs in 2003, ~6,300 deaths in 2016 and ~3.5 billion DALYs in 2016. ref[2] STHs are widely distributed in tropical and subtropical regions, with high infection rates in Sub-Saharan Africa, the Americas, China, and South East Asia. Deworming is recommended by the WHO as a preventive measure for people who are at risk. Deworming treatment includes albendazole and mebendazole for *A lumbricoides, T. trichiura*, and hookworms, and ivermectin for *S. stercoralis*. Although the deworming treatment is very effective, the availability of deworming medication is limited in heavily burdened resource-limited regions.

Current diagnostics

STHs are currently diagnosed by microscopic visualization of parasite eggs or larvae. The Kato-Katz method is the reference diagnostic method[135] that relies on microscopic analysis of stool samples. However, this method has low sensitivity when the worm burden is low, and it requires time, specialized skills and equipment, making it less suitable for daily use in health clinics in resource-limited settings. Currently, DNA detection assays, such as PCR, of parasite DNA in faeces is widely used for epidemiology studies but not for routine diagnosis as it is more expensive than the deworming treatment which is administered on a large scale.[3] Other microscopy methods include Mini-FLOTAC and the Baermann technique. ELISA is also preferred for diagnosing strongyloidiasis. [136]

PoC test

There is a proof-of-principle study for a smart microscopy tool called Kankanet, which is a smartphone equipped with a USB video class microscope attachment. Kankanet is set to recognise *Ascaris lumbricoides, Trichuris trichiura* and hookworms.[137] However, it has been shown that Kankanet only works well for *Ascaris*

lumbricoides when compared to the Kato-Katz method using faecal samples. LAMP based assays and colorimetric isothermal assay (SmartAmp2) to identify hookworm (*N. americanus*) have been developed for screening. [136]

Implementation need

Research to improve the algorithms that recognize the different STH species are needed to further validate the use of smart microscopy tools. A field deployable PoC test that detects specific biomarkers would improve diagnoses in the field. Multiplexed tests would also be beneficial as infections with different STH species are common in endemic regions.[2] Standardized diagnostic procedures and guidelines are required to limit variations in prevalence for more effective epidemiology studies. A field-deployable test to detect resistance to treatment is also urgently needed.[2]

2.6.23 Trachoma

Trachoma is an eye disease that is caused by infection with the bacterium *Chlamydia trachomatis*. It is spread by personal contact (via hand contact, clothes, and sharing linen) and through house flies (*Muscidae*) that come in contact with discharge from the eyes or nose of an infected person. Trachoma causes trichiasis after repeated infections, which occurs when the eyelashes turn inwards and scratch the surface of the eye and permanently damage the cornea. Although trichiasis is not fatal, it is extremely painful and results in irreversible visual impairment and eventual blindness. Trichiasis is the world's leading infectious cause of preventable blindness. Trachoma remains endemic in the global south (Sub-Saharan Africa, and South America) with 142 million people (2019) still at risk of acquiring the infection. Trachoma is treated by MDA of azithromycin, which acts to reduce the ocular reservoir of *C.trachomatis*. Improved access to sanitation to improve facial cleanliness greatly reduces the effects of trachoma. Environmental improvements, such as removal of human faeces from the environment, reduces breeding sites for the vectors (muscid flies).[2] The SAFE strategy is an effective prevention approach (Surgery for infected people, Antibiotic MDA to clear ocular *C.trachomatis*, Facial cleanliness and Environmental improvement to reduce transmission).

Current diagnostics

Trachoma is diagnosed by medical staff and trained healthcare workers. Diagnoses are done empirically with a grading system, based on visible physical symptoms. The WHO provides 5 grading classifications: Trachomatous Inflammation Follicular - which mostly requires topical treatment. Trachomatous Inflammation Intense - during which topical and systemic treatments are considered. Trachomatous Scarring when scars are visible as in the tarsal conjunctiva and which may obscure tarsal blood vessels. Trachomatous trichiasis when an individual is referred for eyelid surgery, and finally Corneal Opacity - a stage during which a person is irreversibly

blind. The greatest challenge is that early infections often do not present visible symptoms. Although not used in practice much, a sample of fluid from the eye can be sent to a lab for testing. PCR or culture methods can then be used, but these methods are too expensive for use in national programmes.[138] There are numerous laboratory-based tests that are useful for low prevalence areas. *C.trachomatis* can be cultured in different cell culture systems. There are cytology tests by Geimsa stain or direct fluorescent antibody tests as well as an enzyme immunoassay that detect antibodies against chlamydial antigens.[139] PoC tests are required to indicate when certain districts need to start MDA interventions before the infection becomes widespread.

PoC test

There is a PoC test for trachoma that detects antibodies against an antigen (pgp3) from *C.trachomatis*.[140] This test works well in the field, but is limited to advanced infections. There is a grading tool that helps healthcare workers to measure the follicle size with a graded adhesive sticker that fits on the graders thumb.[141]

Implementation need

A confirmatory (test-of-cure) PoC test that can detect acute infections is required to determine when MDA can be discontinued in an endemic region.[142]

2.6.24 Yaws

Yaws is a bacterial infection that is caused by the *Treponema pallidum* subspecies *pertenue*, which is closely related to syphilis.[143] This disease is spread by direct skin-to-skin contact with highly infectious lesions. Yaws is characterized by different stages of clinical manifestation. After a variable incubation period (between 10 and 90 days) a primary lesion appears at the patient's infection site, which can develop into a large papilloma (ulcer). Without antibiotic treatment, the patient can develop secondary lesions in a few weeks to 2 years after the primary lesion. Yaws can develop into a chronic stage of infection which results in severe deforming bone lesions. Patients with yaws can also enter a latent stage of infection, which can only be serologically determined and will last for a lifetime. Yaws is endemic in the warm, humid tropical areas of Africa, Asia, Latin America and the Pacific Islands. The most affected group are children in poor rural areas. Approximately 80.000 cases of yaws was reported (2018).ref[2,144,145] Treatment includes the use of two antibiotics, azithromycin or benzathine penicillin.[144] Oral azithromycin is used for total community treatment.[2]

Current diagnostics

Yaws is most commonly diagnosed by treponemal and non-treponemal immunological tests that are also used for syphilis. For treponemal tests, the *Treponema pallidum* particle agglutination assay, *Treponema pallidum* hemagglutination assay

or the fluorescent treponemal antibody test are commonly used. For non-treponemal tests, the Rapid Plasma Reagin or Venereal Disease Research Laboratory assays are commonly used. Treponemal tests are highly specific but cannot distinguish between treated or non-treated Yaws. Non-Treponemal tests are not specific but can detect active infections after treatment. Thus, both immunological tests are needed for an accurate diagnosis.[144,146] Other methods that are used to detect yaws are dark-field microscopy and direct nucleic acid detection using PCR. Dark-field microscopy requires fresh samples of active lesions. Nucleic-acid-based tests can distinguish between syphilis and yaws. However, nucleic-acid-based tests are limited to well-equipped laboratories.[144,147]

PoC test

The Dual Path Platform test (DPP®) can be used as a PoC test to detect both treponemal and a non-treponemal infections. DPP® was tested successfully in China and later tested in a resource-limited settings to determine the healthcare workers and patient's perceptions of the test.[146,148] There is also a LAMP proof-of-principle test that can be applied to target a specific yaws gene in swab samples from lesions. However, this LAMP assay has not been adapted for field-deployability.[149]

Implementation need

A field-deployable nucleic-acid-based PoC test is needed to distinguish between yaws and syphilis in resource-limited settings. A multiplexed test for yaws and syphilis would facilitate effective diagnoses of these diseases.

References

1. Li L, Liu X, Zhou B, Zhang S, Wang G, Ma G, et al. Multiple food-borne trematodiases with profound systemic involvement: a case report and literature review. BMC Infect Dis. 2019 Jun 14;19(1):526.
2. World Health Organization. WHO global consultations for new roadmap on NTDs [Internet]. 2019. 47 p. Available from: https://www.who.int/neglected_diseases/news/WHO-global-consultations-for-new-Roadmap-on-NTD/en/
3. Bergquist R, Johansen MV, Utzinger J. Diagnostic dilemmas in helminthology: what tools to use and when? Trends Parasitol. Elsevier Current Trends; 2009 Apr 1;25(4):151–6.
4. Mubanga C, Mwape KE, Phiri IK, Trevisan C, Zulu G, Chabala C, et al. Progress on the development of rapid diagnostic tests for foodborne neglected zoonotic helminthiases: A systematic review. Acta Tropica. 2019 Jun;194:135–47.
5. Monteiro KJL, Calegar DA, Amazonas Research Group, Carvalho-Costa FA, Jaeger LH. Kato-Katz thick smears as a DNA source of soil-transmitted helminths. J Helminthol. 2018 Nov 15;94:e10.
6. Sadaow L, Sanpool O, Rodpai R, Yamasaki H, Ittiprasert W, Mann VH, et al. Development of an Immunochromatographic Point-of-Care Test for Serodiagnosis of Opisthorchiasis and Clonorchiasis. Am J Trop Med Hyg. The American Society of Tropical Medicine and Hygiene; 2019;101(5):1156–60.
7. Fleury A, Cardenas G, Adalid-Peralta L, Fragoso G, Sciutto E. Immunopathology in Taenia solium neurocysticercosis. Mahanty S, editor. Parasite Immunol. 2016 Mar;38(3):147–57.
8. Garcia HH, Nash TE, Del Brutto OH. Clinical symptoms, diagnosis, and treatment of neurocysticercosis. Lancet Neurol. 2014 Dec;13(12):1202–15.
9. World Health Organization. WHO estimates of the global burden of foodborne diseases: foodborne disease burden epidemiology reference group 2007-2015. 2015.
10. Mohammad IN, Heiner DC, Miller BL, Goldberg MA, Kagan IG. Enzyme-Linked Immunosorbent-Assay for the Diagnosis of Cerebral Cysticercosis. J Clin Microbiol. 1984;20(4):775–9.
11. World Health Organization. Taenia solium taeniasis/cysticercosis diagnostic tools: report of a stakeholder meeting, Geneva, 17-18 December 2015. World Health Organization. 2016.
12. Corstjens PLAM, de Dood CJ, Priest JW, Tanke HJ, Handali S, Cysticercosis Working Group in P. Feasibility of a lateral flow test for neurocysticercosis using novel up-converting nanomaterials and a lightweight strip analyzer. Gabriël S, editor. PLoS Negl Trop Dis. 2014 Jul;8(7):e2944–4.
13. Tamarozzi F, Mariconti M, Covini I, Brunetti E. [Rapid diagnostic tests for the serodiagnosis of human cystic echinococcosis]. Bull Soc Pathol Exot. 2017 Feb;110(1):20–30.
14. Otero-Abad B, Torgerson PR. A Systematic Review of the Epidemiology of Echinococcosis in Domestic and Wild Animals. Garcia HH, editor. Public Library of Science; 2013 Jun;7(6).
15. Schipper HG, Kager PA. Diagnosis and treatment of hepatic echinococcosis: An overview. Scand J Gastroenterol Suppl. Taylor & Francis; 2004;39(241):50–5.
16. Tamarozzi F, Covini I, Mariconti M, Narra R, Tinelli C, De Silvestri A, et al. Comparison of the Diagnostic Accuracy of Three Rapid Tests for the Serodiagnosis of Hepatic Cystic

Echinococcosis in Humans. Hirayama K, editor. PLoS Negl Trop Dis. Public Library of Science; 2016 Feb;10(2).
17. Fooks AR, Cliquet F, Finke S, Freuling C, Hemachudha T, Mani RS, et al. Rabies. Nat Rev Dis Primers. Nature Publishing Group; 2017 Nov 30;3(1):17091–19.
18. Jackson AC. Rabies: a medical perspective. Rev - Off Int Epizoot. 2018 Aug;37(2):569–80.
19. Moore SM. Rabies: Current Preventive Strategies. Vet Clin North Am Small Anim Pract. 2019 Jul;49(4):629–41.
20. Singh R, Singh KP, Cherian S, Saminathan M, Kapoor S, Manjunatha Reddy GB, et al. Rabies - epidemiology, pathogenesis, public health concerns and advances in diagnosis and control: a comprehensive review. Vet Q. 9 ed. 2017 Dec;37(1):212–51.
21. Certoma A, Lunt RA, Vosloo W, Smith I, Colling A, Williams DT, et al. Assessment of a rabies virus rapid diagnostic test for the detection of Australian bat lyssavirus. Trop Med Infect Dis. Multidisciplinary Digital Publishing Institute; 2018 Oct 4;3(4):109.
22. Fooks AR, Banyard AC, Horton DL, Johnson N, McElhinney LM, Jackson AC. Current status of rabies and prospects for elimination. Lancet. 2014;384(9951):1389–99.
23. Elmore SA, Chipman RB, Slate D, Huyvaert KP, VerCauteren KC, Gilbert AT. Management and modeling approaches for controlling raccoon rabies: The road to elimination. Zinsstag J, editor. PLoS Negl Trop Dis. 2017 Mar;11(3).
24. Léchenne M, Naïssengar K, Lepelletier A, Alfaroukh IO, Bourhy H, Zinsstag J, et al. Validation of a Rapid Rabies Diagnostic Tool for Field Surveillance in Developing Countries. Rupprecht CE, editor. PLoS Negl Trop Dis. 2016 Oct;10(10):e0005010.
25. Kang B, Oh J, Lee C, Park B-K, Park Y, Hong K, et al. Evaluation of a rapid immunodiagnostic test kit for rabies virus. J Virol Methods. 2007 Oct;145(1):30–6.
26. Brito AC de, Bittencourt M de JS. Chromoblastomycosis: an etiological, epidemiological, clinical, diagnostic, and treatment update. An Bras Dermatol. 3rd ed. 2018 Jul;93(4):495–506.
27. Queiróz AJR, Pereira Domingos F, Antônio JR. Chromoblastomycosis: clinical experience and review of literature. Int J Dermatol. 2nd ed. 2018 Nov;57(11):1351–5.
28. Hay R, Denning DW, Bonifaz A, Queiroz-Telles F, Beer K, Bustamante B, et al. The Diagnosis of Fungal Neglected Tropical Diseases (Fungal NTDs) and the Role of Investigation and Laboratory Tests: An Expert Consensus Report. Trop Med Infect Dis. Multidisciplinary Digital Publishing Institute; 2019 Dec 1;4(4):122.
29. Bienvenu A-L, Picot S. Mycetoma and Chromoblastomycosis: Perspective for Diagnosis Improvement Using Biomarkers. Molecules. Multidisciplinary Digital Publishing Institute; 2020 Jan 1;25(11):2594.
30. Soriano JB, Banoub JAM, Kanchan T, Yasin YJ. GBD 2017 DALYs and HALE Collaborators. Global, regional, and national disability-adjusted life-years (DALYs) for 359 diseases and injuries and healthy life expectancy (HALE) for 195 countries and territories, 1990-2017: a systematic analysis for the Global Burden of Disease Study (vol 392, pg 1859, 2018). Lancet. 2019;393(10190):E44–4.
31. Markle WH, Makhoul K. Cutaneous leishmaniasis: recognition and treatment. Am Fam Physician. Am Fam Physician; 2004 Mar 15;69(6):1455–60.
32. Schallig HDFH, Hu RVP, Kent AD, van Loenen M, Menting S, Picado A, et al. Evaluation of point of care tests for the diagnosis of cutaneous leishmaniasis in Suriname. BMC Infect Dis. BioMed Central; 2019 Dec 1;19(1):25.
33. Calderon F, Low DE, Ramos AP, Arevalo J, Veland N, Boggild AK, et al. Polymerase Chain

Reaction Detection of Leishmania kDNA from the Urine of Peruvian Patients with Cutaneous and Mucocutaneous Leishmaniasis. Am J Trop Med Hyg. 2011 Apr 5;84(4):556–61.
34. Adams ER, Schoone G, Versteeg I, Gomez MA, Diro E, Mori Y, et al. Development and Evaluation of a Novel Loop-Mediated Isothermal Amplification Assay for Diagnosis of Cutaneous and Visceral Leishmaniasis. Loeffelholz MJ, editor. J Clin Microbiol. American Society for Microbiology Journals; 2018 Jul;56(7):S102.
35. Mirzaei A. Immunodetection and molecular determination of visceral and cutaneous Leishmania infection using patients' urine. Infect Genet Evol. 2018 Sep 1;63:257–68.
36. Veland N, Espinosa D, Valencia BM, Ramos AP, Calderon F, Arevalo J, et al. Polymerase chain reaction detection of Leishmania kDNA from the urine of Peruvian patients with cutaneous and mucocutaneous leishmaniasis. Am J Trop Med Hyg. 2011 Apr;84(4):556–61.
37. Zijlstra EE, van de Sande WWJ, Welsh O, Mahgoub ES, Goodfellow M, Fahal AH. Mycetoma: a unique neglected tropical disease. Lancet Infect Dis. 2016 Jan;16(1):100–12.
38. Emmanuel P, Dumre SP, John S, Karbwang J, Hirayama K. Mycetoma: a clinical dilemma in resource limited settings. Ann Clin Microbiol Antimicrob. 2018;17(1).
39. Verma P, Jha A. Mycetoma: reviewing a neglected disease. Clinical and Experimental Dermatology. 2019 Mar;44(2):123–9.
40. Ahmed AA, van de Sande W, Fahal AH. Mycetoma laboratory diagnosis: Review article. Clements ACA, editor. PLoS Negl Trop Dis. 2017 Aug;11(8):e0005638.
41. DNDi. Mycetoma: New hope for neglected patients - Developing effective treatments for a truly neglected disease. 2019. 12 p.
42. GBD 2016 DALYs and HALE Collaborators. Global, Regional, and National Disability-adjusted Life-years (DALYs) for 333 Diseases and Injuries and Healthy Life Expectancy (HALE) for 195 Countries and Territories, 1990–2016: a Systematic Analysis for the Global Burden of Disease Study 2016. Vol. 390, Lancet. 2017. 85 p.
43. https://www.who.int/health-topics/human-african-trypanosomiasis [Internet]. [cited 2020 Jul 31]. Available from: https://www.who.int
44. Sutherland CS, Stone CM, Steinmann P, Tanner M, Tediosi F. Seeing beyond 2020: an economic evaluation of contemporary and emerging strategies for elimination of Trypanosoma brucei gambiense. Lancet Glob Health. 2017 Jan;5(1):e69–e79.
45. Bonnet J, Boudot C, Courtioux B. Overview of the Diagnostic Methods Used in the Field for Human African Trypanosomiasis: What Could Change in the Next Years? BioMed Research International. Hindawi; 2015;2015(1):583262–10.
46. Biéler S, Waltenberger H, Barrett MP, McCulloch R, Mottram JC, Carrington M, et al. Evaluation of Antigens for Development of a Serological Test for Human African Trypanosomiasis. Tanowitz HB, editor. PLoS One. 2016;11(12):e0168074–.
47. Matovu E, Kazibwe AJ, Mugasa CM, Ndung'u JM, Njiru ZK. Towards Point-of-Care Diagnostic and Staging Tools for Human African Trypanosomiaisis. J Trop Med. Hindawi; 2012;2012(4):340538–9.
48. Converse PJ, Xing Y, Kim KH, Tyagi S, Li S-Y, Almeida DV, et al. Accelerated Detection of Mycolactone Production and Response to Antibiotic Treatment in a Mouse Model of Mycobacterium ulcerans Disease. Phillips RO, editor. PLoS Negl Trop Dis. 2014 Jan 2;8(1):e2618–7.
49. World Health Organization. Management of Buruli ulcer–HIV coinfection. World Health

Organization. 2015 Mar pp. 1–21.
50. Sakyi SA, Aboagye SY, Darko Otchere I, Yeboah-Manu D. Clinical and Laboratory Diagnosis of Buruli Ulcer Disease: A Systematic Review. Can J Infect Dis Med Microbiol. Hindawi; 2016;2016(17):5310718–10.
51. Röltgen K, Cruz I, Ndung'u JM, Pluschke G. Laboratory Diagnosis of Buruli Ulcer: Challenges and Future Perspectives. Vol. 46. Cham (CH): Springer; 2019. 20 p.
52. World Health Organization, diagnostics F. Report of a WHO–FIND meeting on diagnostics for Buruli ulcer. World Health Organization. 2018 Oct pp. 1–19.
53. Diesburg SP, Guelig D, Burton R, Singleton J, Labarre P. Development of a single-use, disposable, electricity-free, nucleic acid amplification platform. GHTC. IEEE; 2015;:400–6.
54. McManus DP, Dunne DW, Sacko M, Utzinger J, Vennervald BJ, Zhou X-N. Schistosomiasis. Nat Rev Dis Primers. Nature Publishing Group; 2018 Aug 9;4(1):13–9.
55. Nelwan ML. Schistosomiasis: Life Cycle, Diagnosis, and Control. Curr Ther Res Clin Exp. 2019;91:5–9.
56. Hinz R, Schwarz NG, Hahn A, Frickmann H. Serological approaches for the diagnosis of schistosomiasis - A review. Mol Cell Probes. 2017 Feb;31:2–21.
57. Hotez PJ, Alvarado M, Basáñez M-G, Bolliger I, Bourne R, Boussinesq M, et al. The Global Burden of Disease Study 2010: Interpretation and Implications for the Neglected Tropical Diseases. de Silva N, editor. PLoS Negl Trop Dis. 2014 Jul 24;8(7):e2865.
58. Utzinger J, Becker SL, Van Lieshout L, Van Dam GJ, Knopp S. New diagnostic tools in schistosomiasis. Clinical Microbiology and Infection. 2015 Jun;21(6):529–42.
59. Verweij JJ, Stensvold CR. Molecular Testing for Clinical Diagnosis and Epidemiological Investigations of Intestinal Parasitic Infections. Clin Microbiol Rev. 2014 Apr;27(2):371–418.
60. Utzinger J, Becker SL, Knopp S, Blum J, Neumayr AL, Keiser J, et al. Neglected tropical diseases: diagnosis, clinical management, treatment and control. Swiss Med Wkly. EMH; 2012;142(4748):w13727.
61. Legesse M, Erko B. Field-based evaluation of a reagent strip test for diagnosis of Schistosoma mansoni by detecting circulating cathodic antigen in urine before and after chemotherapy. Trans R Soc Trop Med Hyg. 2007 Jul;101(7):668–73.
62. Colley DG, King CH, Kittur N, Ramzy RMR, Secor WE, Fredericks-James M, et al. Evaluation, Validation, and Recognition of the Point-of-Care Circulating Cathodic Antigen, Urine-Based Assay for Mapping Schistosoma mansoni Infections. Am J Trop Med Hyg. 2020 Jul 9;103(1_Suppl):42–9.
63. Beltrame A, Guerriero M, Angheben A, Gobbi F, Requena-Mendez A, Zammarchi L, et al. Accuracy of parasitological and immunological tests for the screening of human schistosomiasis in immigrants and refugees from African countries: An approach with Latent Class Analysis. Keiser J, editor. PLoS Negl Trop Dis. 2017 Jun 5;11(6):e0005593–15.
64. Agbana T, Van GY, Oladepo O, Vdovin G, Oyibo W, Diehl JC. Schistoscope - Towards a locally producible smart diagnostic device for Schistosomiasis in Nigeria. GHTC. IEEE; 2019;:1–8.
65. Corstjens PLAM, de Dood CJ, Knopp S, Clements MN, Ortu G, Umulisa I, et al. Circulating Anodic Antigen (CAA): A Highly Sensitive Diagnostic Biomarker to Detect Active Schistosoma Infections—Improvement and Use during SCORE. Am J Trop Med Hyg. 2020 Jul 9;103(1_Suppl):50–7.
66. Rassi A, Marin-Neto JA. Chagas disease. Lancet. 2010 Apr 17;375(9723):1388–402.

67. Coura JR, Viñas PA. Chagas disease: a new worldwide challenge. Nature. Nature Publishing Group; 2010 Jun 1;465(7301):S6–S7.
68. Schijman AG, Vigliano C, Burgos J, Favaloro R, Perrone S, Laguens R, et al. Early diagnosis of recurrence of Trypanosoma cruzi infection by polymerase chain reaction after heart transplantation of a chronic Chagas' heart disease patient. J Heart Lung Transplant. 2000 Nov;19(11):1114–7.
69. Sosa-Estani S, Viotti R, Segura EL. Therapy, diagnosis and prognosis of chronic Chagas disease: insight gained in Argentina. Mem Inst Oswaldo Cruz. Fundação Oswaldo Cruz; 2009 Jul;104 Suppl 1(suppl 1):167–80.
70. Sánchez-Camargo CL, Albajar-Viñas P, Wilkins PP, Nieto J, Leiby DA, Paris L, et al. Comparative evaluation of 11 commercialized rapid diagnostic tests for detecting Trypanosoma cruzi antibodies in serum banks in areas of endemicity and nonendemicity. Gilligan PH, editor. J Clin Microbiol. American Society for Microbiology Journals; 2014 Jul;52(7):2506–12.
71. Shah V, Ferrufino L, Gilman RH, Ramirez M, Saenza E, Malaga E, et al. Field Evaluation of the InBios Chagas Detect Plus Rapid Test in Serum and Whole-Blood Specimens in Bolivia. Wilkins PP, editor. Clin Vaccine Immunol. American Society for Microbiology; 2014 Dec;21(12):1645–9.
72. Ponce C, Ponce E, Vinelli E, Montoya A, de Aguilar V, Gonzalez A, et al. Validation of a Rapid and Reliable Test for Diagnosis of Chagas' Disease by Detection of Trypanosoma cruzi-Specific Antibodies in Blood of Donors and Patients in Central America. J Clin Microbiol. American Society for Microbiology Journals; 2005 Oct 1;43(10):5065–8.
73. Gideon Informatics, Inc., Berger DS. Visceral Leishmaniasis: Global Status. GIDEON Informatics Inc; 2019.
74. Meeting WECOTCOTL, World Health Organization. Control of the Leishmaniases. 2010. 1 p.
75. CDC. Parasites - Leishmaniasis. [Internet]. [cited 2020 Jul 31]. Available from: https://www.cdc.gov/parasites/leishmaniasis/index.html
76. Tlamcani Z. Visceral leishmaniasis: an update of laboratory diagnosis. Asian Pacific Journal of Tropical Disease. 2016 Jul 1;6(7):505–8.
77. Sakkas H, Gartzonika C, Levidiotou S. Laboratory diagnosis of human visceral leishmaniasis. J Vector Borne Dis. 2016 Mar;53(1):8–16.
78. Akhoundi M, Downing T, Votýpka J, Kuhls K, Lukeš J, Cannet A, et al. Leishmania infections: Molecular targets and diagnosis. Molecular Aspects of Medicine. Elsevier Ltd; 2017 Oct 1;57:1–29.
79. Adams ER, Jacquet D, Schoone G, Gidwani K, Boelaert M, Cunningham J. Leishmaniasis direct agglutination test: using pictorials as training materials to reduce inter-reader variability and improve accuracy. Büscher P, editor. PLoS Negl Trop Dis. Public Library of Science; 2012;6(12):e1946.
80. Boelaert M, Verdonck K, Menten J, Sunyoto T, van Griensven J, Chappuis F, et al. Rapid tests for the diagnosis of visceral leishmaniasis in patients with suspected disease. Cochrane Infectious Diseases Group, editor. Cochrane Database Syst Rev. John Wiley & Sons, Ltd; 2014;2014(6).
81. Singh OP, Sundar S. Developments in diagnosis of visceral leishmaniasis in the elimination era. J Parasitol Res. Hindawi; 2015 Jan 1;2015:239469.
82. Vaish M, Mehrotra S, Chakravarty J, Sundar S. Noninvasive molecular diagnosis of hu-

man visceral leishmaniasis. J Clin Microbiol. 2011 May;49(5):2003–5.
83. Vaish M, Singh OP, Chakravarty J, Sundar S. rK39 antigen for the diagnosis of visceral leishmaniasis by using human saliva. Am J Trop Med Hyg. The American Society of Tropical Medicine and Hygiene; 2012 Apr;86(4):598–600.
84. Bezuneh A, Mukhtar M, Abdoun A, Teferi T, Takele Y, Diro E, et al. Comparison of point-of-care tests for the rapid diagnosis of visceral leishmaniasis in East African patients. Am J Trop Med Hyg. The American Society of Tropical Medicine and Hygiene; 2014 Dec;91(6):1109–15.
85. Humbert MV, Costa LE, Katis I, Fonseca Ramos F, Sanchéz Machado A, Sones C, et al. A rapid diagnostic test for human Visceral Leishmaniasis using novel Leishmania antigens in a Laser Direct-Write Lateral Flow Device. Emerg Microbes Infect. Taylor & Francis; 2019;8(1):1178–85.
86. Matlashewski G, Das VNR, Pandey K, Singh D, Das S, Ghosh AK, et al. Diagnosis of visceral leishmaniasis in Bihar India: comparison of the rK39 rapid diagnostic test on whole blood versus serum. Debrabant A, editor. PLoS Negl Trop Dis. Public Library of Science; 2013;7(5):e2233.
87. Mondal D, Ghosh P, Khan MAA, Hossain F, Böhlken-Fascher S, Matlashewski G, et al. Mobile suitcase laboratory for rapid detection of Leishmania donovani using recombinase polymerase amplification assay. Parasites & Vectors. Parasites & Vectors; 2016 May 13;9(1):281–8.
88. Koltas IS, Eroglu F, Uzun S, Alabaz D. A comparative analysis of different molecular targets using PCR for diagnosis of old world leishmaniasis. Experimental Parasitology. Elsevier Inc; 2016 May 1;164(C):43–8.
89. Taylor MJ, Hoerauf A, Bockarie M. Lymphatic filariasis and onchocerciasis. Lancet. 2010;376(9747):1175–85.
90. CDC. Parasites - Lymphatic Filariasis. [Internet]. [cited 2020 Jul 31]. Available from: https://www.cdc.gov/parasites/lymphaticfilariasis/index.html
91. Filariasis WECO. Lymphatic filariasis. 1984. 1 p.
92. Rebollo MP, Bockarie MJ. Can Lymphatic Filariasis Be Eliminated by 2020? Trends Parasitol. 2017 Feb;33(2):83–92.
93. Hertz MI, Nana-Djeunga H, Kamgno J, Jelil Njouendou A, Chawa Chunda V, Wanji S, et al. Identification and characterization of Loa loa antigens responsible for cross-reactivity with rapid diagnostic tests for lymphatic filariasis. Specht S, editor. PLoS Negl Trop Dis. 2018 Nov;12(11):e0006963.
94. Natrajan MS, Rojas A, Waggoner JJ. Beyond Fever and Pain: Diagnostic Methods for Chikungunya Virus. Kraft CS, editor. J Clin Microbiol. American Society for Microbiology Journals; 2019 Jun;57(6):209.
95. Bloch D. The Cost And Burden Of Chikungunya In The Americas. 2019. pp. 1–34.
96. CDC. Chikungunya Virus. [Internet]. [cited 2020 Jul 31]. Available from: https://www.cdc.gov/chikungunya/index.html
97. Hayashida K, Orba Y, Sequeira PC, Sugimoto C, Hall WW, Eshita Y, et al. Field diagnosis and genotyping of chikungunya virus using a dried reverse transcription loop-mediated isothermal amplification (LAMP) assay and MinION sequencing. Kittayapong P, editor. PLoS Negl Trop Dis. 2019 Jun 1;13(6):e0007480.
98. Vongsouvath M, Bharucha T, Seephonelee M, de Lamballerie X, Newton PN, Dubot-Pérès A. Harnessing Dengue Rapid Diagnostic Tests for the Combined Surveillance

of Dengue, Zika, and Chikungunya Viruses in Laos. Am J Trop Med Hyg. The American Society of Tropical Medicine and Hygiene; 2020 Jun;102(6):1244–8.
99. Johnston G, Sladden M. Scabies: diagnosis and treatment. BMJ. 2005 Sep 17;331(7517):619–22.
100. Kinyanjui T, Middleton J, Güttel S, Cassell J, Ross J, House T. Scabies in residential care homes: Modelling, inference and interventions for well-connected population sub-units. Alizon S, editor. PLoS Comput Biol. 2018 Mar;14(3):e1006046.
101. Soriano JB, Banoub JAM, Kanchan T, Yasin YJ. GBD 2017 DALYs and HALE Collaborators. Global, regional, and national disability-adjusted life-years (DALYs) for 359 diseases and injuries and healthy life expectancy (HALE) for 195 countries and territories, 1990-2017: a systematic analysis for the Global Burden of Disease Study (vol 392, pg 1859, 2018). Lancet. 2019;393(10190):E44–4.
102. Leung V, Miller M. Detection of scabies: A systematic review of diagnostic methods.Canadian Journal of Infectious Diseases and Medical Microbiology. Hindawi; 2011;22(4):143–6.
103. Engelman D, Fuller LC, Steer AC, Scabies IAC. Consensus criteria for the diagnosis of scabies: A Delphi study of international experts. Vinetz JM, editor. PLoS Negl Trop Dis. Public Library of Science; 2018 May;12(5).
104. World Health Organization. Ending the neglect to attain the sustainable development goals: a road map for neglected tropical diseases 2021–2030. WHO. 2020.
105. Park JH, Kim CW, Kim SS. The diagnostic accuracy of dermoscopy for scabies. Ann Dermatol. 2012 May;24(2):194–9.
106. Colebunders R, Stolk WA, Siewe Fodjo JN, Mackenzie CD, Hopkins A. Elimination of onchocerciasis in Africa by 2025: an ambitious target requires ambitious interventions. Infect Dis Poverty. 2019 Oct 3;8(1):83.
107. Puri PK. Onchocerciasis. Dermatopathology Diagnosis. 2015 Mar 5;:1–3.
108. Gideon Informatics, Inc., Berger DS. Onchocerciasis: Global Status. GIDEON Informatics Inc; 2020.
109. Hopkins A. Onchocerciasis: the beginning of the end? Community Eye Health. 2019;31(104):94.
110. Unnasch TR, Golden A, Cama V, Cantey PT. Diagnostics for onchocerciasis in the era of elimination. International Health. 2018 Feb 19;10(suppl_1):i20–6.
111. Steel C, Golden A, Stevens E, Yokobe L, Domingo GJ, de los Santos T, et al. Rapid Point-of-Contact Tool for Mapping and Integrated Surveillance of Wuchereria bancrofti and Onchocerca volvulus Infection. Wilkins PP, editor. Clin Vaccine Immunol. American Society for Microbiology; 2015 Aug;22(8):896–901.
112. World Health Organization. Human African trypanosomiasis (Sleeping sickness) [Internet]. https://www.who.int/health-topics/human-african-trypanosomiasis. [cited 2020 Jul 31].
113. Sternberg JM, Gierliński M, Biéler S, Ferguson MAJ, Ndung'u JM. Evaluation of the diagnostic accuracy of prototype rapid tests for human African trypanosomiasis. Raper J, editor. PLoS Negl Trop Dis. 2014 Dec;8(12):e3373.
114. Lumbala C, Biéler S, Kayembe S, Makabuza J, Ongarello S, Ndung'u JM. Prospective evaluation of a rapid diagnostic test for Trypanosoma brucei gambiense infection developed using recombinant antigens. Caljon G, editor. PLoS Negl Trop Dis. Public Library of Science; 2018 Mar 28;12(3):e0006386.

115. Boelaert M, Mukendi D, Bottieau E, Kalo Lilo JR, Verdonck K, Minikulu L, et al. A Phase III Diagnostic Accuracy Study of a Rapid Diagnostic Test for Diagnosis of Second-Stage Human African Trypanosomiasis in the Democratic Republic of the Congo. EBioMedicine. Elsevier; 2018 Jan 1;27:11–7.
116. Sullivan L, Fleming J, Sastry L, Mehlert A, Wall SJ, Ferguson MAJ. Identification of sVSG117 as an immunodiagnostic antigen and evaluation of a dual-antigen lateral flow test for the diagnosis of human African trypanosomiasis. Raper J, editor. PLoS Negl Trop Dis. Public Library of Science; 2014 Jul;8(7):e2976.
117. Benjamin JM, Abo BN, Brandehoff N. Review Article: Snake Envenomation in Africa. Current Tropical Medicine Reports. 9 ed. Springer International Publishing; 2020 Mar 1;7(1):1–10.
118. Williams HF, Layfield HJ, Vallance T, Patel K, Bicknell AB, Trim SA, et al. The Urgent Need to Develop Novel Strategies for the Diagnosis and Treatment of Snakebites. Toxins (Basel). Multidisciplinary Digital Publishing Institute; 2019 Jun 20;11(6):363.
119. Williams HF, Layfield HJ, Vallance T, Patel K, Bicknell AB, Trim SA, et al. The Urgent Need to Develop Novel Strategies for the Diagnosis and Treatment of Snakebites. Toxins (Basel). 2019 Jun 20;11(6):363.
120. Senji Laxme RR, Khochare S, de Souza HF, Ahuja B, Suranse V, Martin G, et al. Beyond the “big four”: Venom profiling of the medically important yet neglected Indian snakes reveals disturbing antivenom deficiencies. BILLIALD P, editor. PLoS Negl Trop Dis. 2019 Dec;13(12):e0007899.
121. O’Rourke KM, Correlje E, Martin CL, Robertson JD, Isbister GK. Point-of-care derived INR does not reliably detect significant coagulopathy following Australian snakebite. Thromb Res. 2013 Nov;132(5):610–3.
122. Wilder-Smith A, Ooi E-E, Horstick O, Wills B. Dengue. Lancet. 2019;393(10169):350–63.
123. Beatty ME, Beutels P, Meltzer MI, Shepard DS, Hombach J, Hutubessy R, et al. Health economics of dengue: a systematic literature review and expert panel's assessment. Am J Trop Med Hyg. 2011 Mar;84(3):473–88.
124. CDC. Dengue [Internet]. [cited 2020 Jul 31]. Available from: https://www.cdc.gov/dengue/index.html
125. Gan VC, Tan L-K, Lye DC, Pok K-Y, Mok S-Q, Chua RC-R, et al. Diagnosing dengue at the point-of-care: utility of a rapid combined diagnostic kit in Singapore. Bausch DG, editor. PLoS One. 2014;9(3):e90037.
126. Wang SM, Sekaran SD. Early diagnosis of Dengue infection using a commercial Dengue Duo rapid test kit for the detection of NS1, IGM, and IGG. Am J Trop Med Hyg. 2010 Sep;83(3):690–5.
127. Blacksell SD, Jarman RG, Bailey MS, Tanganuchitcharnchai A, Jenjaroen K, Gibbons RV, et al. Evaluation of six commercial point-of-care tests for diagnosis of acute dengue infections: the need for combining NS1 antigen and IgM/IgG antibody detection to achieve acceptable levels of accuracy. Clin Vaccine Immunol. 2011 Dec;18(12):2095–101.
128. Buyon L, Slaven R, Emerson PM, King J, Debrah O, Aboe A, et al. Achieving the endgame: Integrated NTD case searches. Ngondi JM, editor. PLoS Negl Trop Dis. 2018 Dec;12(12):e0006623.
129. Cairncross S, Muller R, Zagaria N. Dracunculiasis (Guinea worm disease) and the eradication initiative. Clin Microbiol Rev. 2002 Apr;15(2):223–46.
130. Alemu Belachew W, Naafs B. Position statement: Leprosy: Diagnosis, treatment and

follow-up. J Eur Acad Dermatol Venereol. 2019 Jul;33(7):1205–13.
131. Fischer M. Leprosy – an overview of clinical features, diagnosis, and treatment. J Dtsch Dermatol Ges [Internet]. John Wiley & Sons, Ltd; 2017 Aug 1;15(8):801–27. Available from: https://onlinelibrary.wiley.com/doi/full/10.1111/ddg.13301
132. Mitra AK, Mawson AR. Neglected Tropical Diseases: Epidemiology and Global Burden. Trop Med Infect Dis. 2017 Aug 5;2(3):36.
133. Reibel F, Cambau E, Aubry A. Update on the epidemiology, diagnosis, and treatment of leprosy. Med Mal Infect [Internet]. Elsevier Masson; 2015 Sep 1;45(9):383–93. Available from: http://www.sciencedirect.com/science/article/pii/S0399077X1500181X
134. van Hooij A, van den Eeden S, Richardus R, Tjon Kon Fat E, Wilson L, Franken KLMC, et al. Application of new host biomarker profiles in quantitative point-of-care tests facilitates leprosy diagnosis in the field. EBioMedicine. 2019 Sep;47:301–8.
135. Cools P, Vlaminck J, Albonico M, Ame S, Ayana M, José Antonio BP, et al. Diagnostic performance of a single and duplicate Kato-Katz, Mini-FLOTAC, FECPAKG2 and qPCR for the detection and quantification of soil-transmitted helminths in three endemic countries. Freeman MC, editor. PLoS Negl Trop Dis. 2019 Aug;13(8):e0007446.
136. Mbong Ngwese M, Prince Manouana G, Nguema Moure PA, Ramharter M, Esen M, Adégnika AA. Diagnostic Techniques of Soil-Transmitted Helminths: Impact on Control Measures. Trop Med Infect Dis. 2020 Jun;5(2):93–17.
137. Yang A, Bakhtari N, Langdon-Embry L, Redwood E, Grandjean Lapierre S, Rakotomanga P, et al. Kankanet: An artificial neural network-based object detection smartphone application and mobile microscope as a point-of-care diagnostic aid for soil-transmitted helminthiases. Bennuru S, editor. PLoS Negl Trop Dis. Public Library of Science; 2019 Aug 5;13(8):e0007577–16.
138. Solomon AW, Holland MJ, Alexander NDE, Massae PA, Aguirre A, Natividad-Sancho A, et al. Mass treatment with single-dose azithromycin for trachoma. N Engl J Med. 2004 Nov 4;351(19):1962–71.
139. Mohammadpour M, Abrishami M, Masoumi A, Hashemi H. Trachoma: Past, present and future. Curr Adv Ophthalmol. 2016 Dec;28(4):165–9.
140. Gwyn S, Mitchell A, Dean D, Mkocha H, Handali S, Martin DL. Lateral flow-based antibody testing for Chlamydia trachomatis. J Immunol Methods. 2016 Aug;435:27–31.
141. Solomon AW, Le Mesurier RT, Williams WJ. A diagnostic instrument to help field graders evaluate active trachoma. Ophthalmic Epidemiol. 2018 Dec;25(5-6):399–402.
142. Harding-Esch EM, Holland MJ, Schémann J-F, Molina S, Sarr I, Andreasen AA, et al. Diagnostic accuracy of a prototype point-of-care test for ocular Chlamydia trachomatis under field conditions in The Gambia and Senegal. Schachter J, editor. PLoS Negl Trop Dis. 2011 Aug;5(8):e1234.
143. Mitjà O, Asiedu K, Mabey D. Yaws. Lancet [Internet]. 2013 Mar;381(9868):763–73. Available from: http://www.sciencedirect.com/science/article/pii/S0140673612621308
144. Giacani L, Lukehart SA. The Endemic Treponematoses. Clin Microbiol Rev. 18 ed. 2014 Jan;27(1):89–115.
145. Fitzpatrick C, Asiedu K, Jannin J. Where the road ends, yaws begins? The cost-effectiveness of eradication versus more roads. Lukehart S, editor. PLoS Negl Trop Dis. 2014 Sep;8(9):e3165.
146. Marks M, Goncalves A, Vahi V, Sokana O, Puiahi E, Zhang Z, et al. Evaluation of a rapid diagnostic test for yaws infection in a community surveillance setting. Phillips RO, edi-

tor. PLoS Negl Trop Dis. 2014 Sep;8(9):e3156.
147. Chi K-H, Danavall D, Taleo F, Pillay A, Ye T, Nachamkin E, et al. Molecular Differentiation of Treponema pallidum Subspecies in Skin Ulceration Clinically Suspected as Yaws in Vanuatu Using Real-Time Multiplex PCR and Serological Methods. Am J Trop Med Hyg. 2015 Jan;92(1):134–8.
148. Yin Y-P, Chen X-S, Wei W-H, Gong K-L, Cao W-L, Yong G, et al. A Dual Point-of-Care Test Shows Good Performance in Simultaneously Detecting Nontreponemal and Treponemal Antibodies in Patients With Syphilis: A Multisite Evaluation Study in China. Clin Infect Dis. 2013;56(5):659–65.
149. Basing LAW, Simpson SV, Adu-Sarkodie Y, Linnes JC. A Loop-Mediated Isothermal Amplification Assay for the Detection of Treponema pallidum subsp. pertenue. Am J Trop Med Hyg. 2020 Apr 20;:tpmd190243

3

Matching Development of Novel Point-of-Care Diagnostic Tests to the Local Context: A Case Study of Visceral Leishmaniasis in Kenya and Uganda

The rapid growth of point-of-care (POC) diagnostic tests necessitates a clear vision of when, where, and why a new POC diagnostic test needs to be developed and how it can be used in a way that matches a local health care context. Here, we present an innovative approach toward developing a concept target product profile (CTPP), which is a new mapping tool that helps researchers match a new diagnostic test to a specific local health care context early in the research and development process. As a case study, we focus on the diagnosis of visceral leishmaniasis (VL) in rural resource-limited regions of Kenya and Uganda. Our stepwise approach integrates elements of design thinking and uses a combination of literature reviews and field research for a context analysis of local health care systems and practices. We then use visual thinking in the form of Gigamaps and patient journeys to identify use case scenarios and to present our findings from the field research to key stakeholders. The use case scenarios describes the diagnostic scope of a new POC test based on the feasibility of the new test, the local need, and the contextual fit. For our case study of VL, we identify 2 valuable use case scenarios, namely test-of-cure and screening and confirmation, and we formulate a CTPP. We anticipate that a CTPP will enable researchers to match a new POC diagnostic test during the research and development process to the local health care context in which it will be used.

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3.1 Introduction

Within the past decade, point-of-care (POC) diagnostic tests have received immense attention[1] because their accuracy and ease of use create the ideal solution for early diagnostics of infectious diseases in resource-limited settings. POC diagnostic tests should involve a minimum number of steps to obtain a real-time result that is easy to interpret. Importantly, POC tests are designed to be performed near or at the site where the patient is to enable a short turnaround time for them to receive treatment and care.[2] Given these favorable characteristics, novel POC technologies for decentralized diagnostics are being developed worldwide at a rapid rate.[3] Ample research opportunities exist for continued development of POC diagnostic tests, particularly for neglected tropical diseases (NTDs), which are a group of chronic, disabling, and potentially fatal diseases that are prevalent in tropical and subtropical regions.[4] NTDs occur predominantly in resource-limited settings, which are defined by the World Bank as resource-constrained (human, environmental, economical) regions with limited infrastructure and/or basic services in low- or middle-income countries.[5] By definition, NTDs receive little attention, and 14 of the 24 NTDs that are currently acknowledged by the World Health Organization (WHO) lack essential POC diagnostic tests.[6]

It is crucial to ensure that new POC diagnostic tests meet the needs of the (multiple) end users and fit constraints within local health care contexts.[7,8] Particularly for resource-limited settings, innovative approaches are required to meet the demands for diagnostics that are suitable for use at the lowest and most constrained level of the health care system. Current POC diagnostic tests are often not compatible with the most constrained level of the health care systems in resource-limited settings because they still require resources, such as a cold chain to store reagents or trained users who are generally not available in such settings. Although these resources are available at higher levels of the health care system (reference laboratories and hospitals), they are not available in the local clinics in remote areas where patients first seek health care. This situation necessitates new POC diagnostic tests that do not depend on additional resources and can be administered by users without extensive training.

Multiple guidelines are available that can be used to jointly guide the design of new POC diagnostic tests that address the needs of the end users and stakeholders in a local health care context. WHO developed the ASSURED criteria (i.e., a diagnostic test should be affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free, and deliverable to end users) to guide the development of new medical technologies and to encourage the adaptation of existing technologies to suit resource-limited settings better. However, the ASSURED criteria are rather general and broadly applicable.[9] WHO and other organizations, such as the Drugs for

Neglected Diseases initiative and the Foundation for Innovative New Diagnostics, collaborate with various stakeholders and experts to develop target product profiles (TPPs), which are more specific guidelines that provide details on the minimal and optimal performance and operational features of diagnostic tests. TPPs are the end result of several rounds of discussions to reach a consensus from policy makers,[10] which is an important yet labor-intensive process. TPPs are developed when the use cases are already defined and it is known when, where, and why the test will be used,[11,12] that is, when specifications have been determined regarding the diagnostic moment (when the patient gets tested), the diagnostic setting (where the patient gets tested), and the purpose of the test (why the patient gets tested), which could be screening and confirmation.

While developing a novel POC diagnostic test, researchers would benefit from having guidelines that are less abstract than the ASSURED criteria, which may be too broad to be effective for a specific context and disease but are less involved and less prescriptive than a full TPP. Indeed, a POC diagnostic test that does not meet all the stringent requirements of a TPP can still be of great value, depending on the local health care context and the needs of the end users.[13] For example, if a TPP defines a desired sensitivity of a test, but a new test is somewhat less sensitive (and thus would be excluded by the TPP) yet is much more stable at higher temperatures or an order of magnitude cheaper, it could still be valuable for a specific disease in an endemic tropical region.[13] To facilitate the design of a POC diagnostic test that fits a health care context, we conclude that an efficient and effective mapping tool is needed, particularly when the diagnostic moment, setting, and purpose of the test are not yet defined.

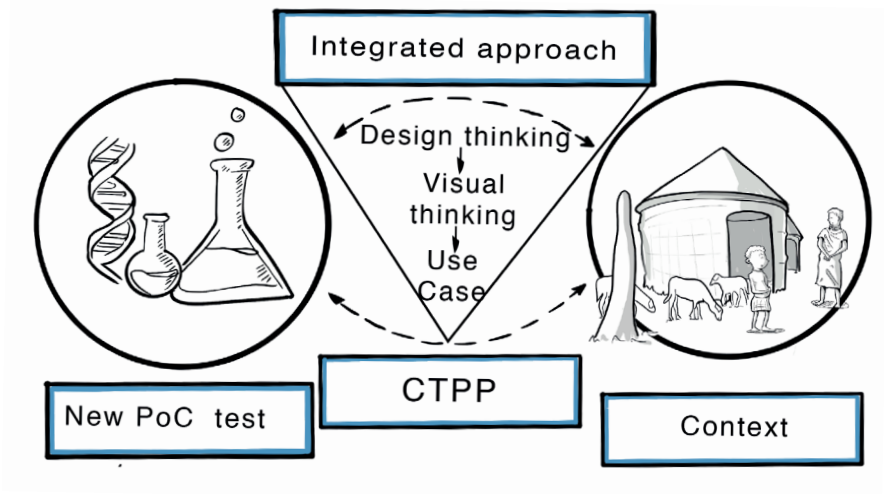

Figure 3.1: Schematic Representation of a CTPP That Bridges the Gap Between a New POC Test and the Local Health Care Context. Abbreviations: CTPP, concept target product profile; POC, point-of-care.

To address this need, we propose to formulate a concept target product profile (CTPP) as an intermediate guideline for developing a diagnostic test that addresses the needs of the end users in the local health care context. A CTPP does not replace a TPP, and it best serves as an intermediate guideline for researchers in the form of a mapping tool. Such a CTPP should preferably be developed at the onset of the research and development (R&D) of a new POC diagnostic test, aiding researchers in considering the context at an early stage while the POC diagnostic test is designed and developed. A CTPP would enable researchers to identify the minimum features for successful implementation of a POC diagnostic test in a local health care context, and it will cost considerably less time and resources than the development of a TPP. To illustrate, a recent TPP for dermal leishmaniases took approximately 4 years and 82 experts to reach a consensus,[7] whereas a CTPP as proposed in this study can take 6 months and a considerably smaller team depending on the interdisciplinary team of choice. Although a CTPP will serve a clear purpose, a full TPP is still required because it is extremely valuable for experts such as policymakers and provides guidelines for further test development by technical experts. In this paper, we propose a step-by-step approach, which includes elements of design thinking,[14] toward developing a CTPP that matches the new POC diagnostic test and the local health care context in which it will be used (Figure 3.1).

3.2 Methodology

For the development of a CTTP, we adopted a stepwise approach, using design-thinking principles (Box). Design thinking is a human-centered approach to innovation that integrates the needs of people (desirability), the possibilities of the technology (feasibility), and the requirements for business success (viability). Design thinking encourages novel and thorough solutions. In general, design thinking is utilized to evaluate the current situation, to identify the actual problem, and thereby provide a guideline for developing customized solutions. Core principles of design thinking are to empathize, define, ideate, prototype, and test.[15] The first 3 of these principles have been integrated into our stepwise approach for formulating a CTPP.

In our case, the design-thinking principles were used to evaluate current diagnostic practices for a disease, identify the diagnostic need for a disease, and provide a diagnostic solution. The approach started with a comprehensive literature survey, followed by observations and semistructured interviews with the health care providers in the field and scientific researchers. In this step, the design principle empathize was integrated to gain an empathetic understanding of the problem at hand. Thereafter, the design-thinking principle define was utilized to combine the information gathered and identify the problem at hand. This step used a set of design tools known as “visual thinking.”[16,17,18] Specifically, “Gigamaps” were used to describe the health care system and define the current diagnostic need. Gigamaps

are large and information-dense diagrams that act as a bridge between inquiry, design, and implementation.[18] Using these, researchers are intentionally encouraged to identify and subsequently use patterns that emerge from the field observations and data.[19] Thereafter, visual depictions of the patient journeys were constructed based on the information gathered from literature and field research. The term patient journey refers to the experiences and processes that a patient goes through during the course of a disease and its treatment.[20,21] These patient journeys provide a detailed yet simplified overview of the challenges faced by the patients while seeking effective diagnoses and subsequent treatment. Finally, the design-thinking principle ideate was applied to obtain the logical solution for the diagnostic needs defined in the previous step. Again, a visual thinking tool in the form of use cases, referred to as scenarios, was utilized to present the complex problem and logical solution. Based on the outcomes from our approach, a CTPP was formulated that integrated a desirable, feasible, and viable solution to a complex societal problem.

The above methodology is expected to be broadly applicable in the field of diagnostics for use in endemic resource-limited settings, since context analysis is always essential to guide the design process during R&D. Developing a CTPP requires the following seven steps.

Step 1: Literature Review

A critical review of the existing literature on a disease and its relevant health care context is performed to gauge the disease endemicity, resource availability, and current diagnostic practices in the resource-constrained settings. Such critical analyses help in identifying the potential and the limitations of the existing diagnostic practices and in identifying stakeholders such as patients, health care staff, patient families, health care organizations, nongovernmental organizations, and government bodies, and thereby highlight the implementation needs for novel diagnostic solutions.

Step 2: Selection of an Endemic Resource-Limited Region for a Case Study

Beyond mere literature study, it is important to direct field research for a case study. Selection criteria for a fitting case should include (i) an endemic region that bears a high burden of a disease; (ii) an area that has an urgent need for POC diagnostic tests; and (iii) a politically stable conflict-free zone for safety and logistic capacity (roads and access to remote endemic areas) for field research.

Step 3: Field Research With Direct Observations and Interviews With Stakeholders

Despite an extensive literature survey, many aspects of diagnoses in remote endemic regions often remain unclear, such as the clinical algorithm for disease identification, the patient's journey from becoming infected to getting treatment, the availability of laboratory equipment, and the level of trained personnel at the lowest most constrained level of the health care system. To obtain observations from the field for filling the knowledge gaps in the literature, a field trip to the selected endemic regions was necessary. The objective of such a field trip is to gather direct observations at various health care levels in that region and to carry out semistructured interviews in the field to obtain expert input from key stakeholders as identified from the literature survey and from advice of the locals.

Step 4: Create Gigamaps and Patient Journeys Based on Insights Gathered

To get a deeper understanding of the diagnostic practices for a disease, the assumptions made by the detailed literature survey (step 1) are analyzed in conjunction with the information that was obtained from semistructured interviews with the health care professionals (step 3). The observations are processed and organized to highlight the limitations of existing diagnostic practices. Since the health care context of a disease in resource-limited settings is complex with social and technical challenges and involves a wide range of stakeholders, understanding the corresponding diagnostic practices for a disease can be quite challenging. To deal with the complexity of such a multifaceted health care system, visual thinking tools are used to present the complex diagnostic problem at hand (define) and to identify the logical solution (ideate) in the form of use case scenarios. Visual Gigamaps are created to outline current diagnostic practices at various stages of the disease, and health care-seeking behaviors of patients from a health care provider's point of view across different health care levels. Next, visual patient journeys are constructed within the selected disease endemic region. In our approach, these extended patient journeys represent a sequence of interactions between the patient, the health care system, and the stakeholders involved, and they consider both the technical, economic, and social factors.

Step 5: Create Use Case Scenarios

Next, we create various scenarios for the application of a diagnostic test within the selected region. Again, visual thinking is utilized to present every scenario to provide a clear understanding of the requisite diagnostic need and to suggest logical solutions.

Step 6: Validation of the Use Case Scenarios

Visualizations of the scenarios from step 5 are used to facilitate discussions with

different stakeholders with an objective to critically select and thus validate the most valuable scenario through which a diagnostic test can meet the local needs of the end users and stakeholders. Thus, detailed discussions are conducted with the stakeholders on each scenario to obtain the most pressing diagnostic need and to define the priorities from the perspective of health care providers.

Step 7: Formulation of a CTPP

Finally, the stepwise approach collectively leads to the formulation of a CTPP for a diagnostic test that will be most effective for the selected disease-endemic region. The CTPP is formulated for the most urgent diagnostic need as viewed from the perspective of health care providers.

3.3 Results of a case study: developing a CTPP for a PoC diagnostic test for VL in Kenya and Uganda

For proof of principle, we selected the NTD visceral leishmaniasis (VL) as a case study to validate our CTPP design approach. Below we explicate the above 7 steps for this case study.

3.3.1 Literature Review on VL Endemicity and VL Health Care Context

Leishmaniases are caused by more than 20 different *Leishmania* species, which are parasites that can be transmitted to humans and other animals by the bite of infected female phlebotomine sand flies.[22,23] Worldwide, there are approximately 2 million new cases each year, and 556 million people are at risk of acquiring the infection.[24] VL (also known as Kala-azar) is the second-largest parasitic killer after malaria[25,26] and the most severe form of leishmaniasis because it affects the visceral organs, particularly the liver, spleen, and lymph nodes.[27] Although VL is curable, it remains a fatal disease because it is often left untreated due to its low index of suspicion by health care providers, late diagnosis, and inadequate case management, especially at an early stage in low-resource settings.[28] Furthermore, the initial symptoms of VL (e.g., persistent fever, weight loss, fatigue, and anemia) overlap with other febrile illnesses, such as malaria, and hence it is often misdiagnosed and treated incorrectly. If left untreated, VL is fatal within 2 years, due to severe anemia or secondary bacterial infections.[29] Furthermore, post-kala-azar dermal leishmaniasis (PKDL) is a skin condition that occurs after VL treatment due to persisting parasites in the skin.[29] Incomplete treatment is a major risk factor for PKDL.[29] Although PKDL lesions are typically self-healing, they pose as infectious reservoirs for sandflies[29] and cause aesthetic and psychological complications that affect the patient's quality of life, especially young adults. VL is a complex disease that is further complicated

by coinfections such as HIV.[28,30,31] Individuals with HIV who are immunosuppressed often present with more severe VL symptoms and require different treatment regimens.[31] Other common coinfections in VL endemic regions include malaria[32] and tuberculosis.[33] Thus VL needs to be diagnosed and managed on a case-by-case basis due to confounding conditions such as immunosuppression.[13]

VL is currently diagnosed by using either one or a combination of the following: (1) empirical clinical observations,[31] (2) immunological rapid diagnostic tests (RDTs) and/or immunoassays, (3) molecular tests to detect the pathogen's DNA in clinical samples, and (4) parasitological tests that require microscopic analyses of invasive splenic or bone marrow aspirations[34,31] (Figure 3.2). Except for clinical methods and RDTs, which can be less reliable, these diagnostic practices require expensive instruments, a stable source of electricity, a well-equipped laboratory, and an expert to operate; therefore, they are inadequate for use within resource-limited settings.[35,30] The most readily used test for VL diagnosis, especially in remote settings, is the rK39 RDT.[36] However, since the rK39 RDT is an immunological test, it is less reliable because the sensitivity of the test differs between individuals,[29,37] and it cannot serve as a test-of-cure due to persisting antibodies after treatment.[30,38]

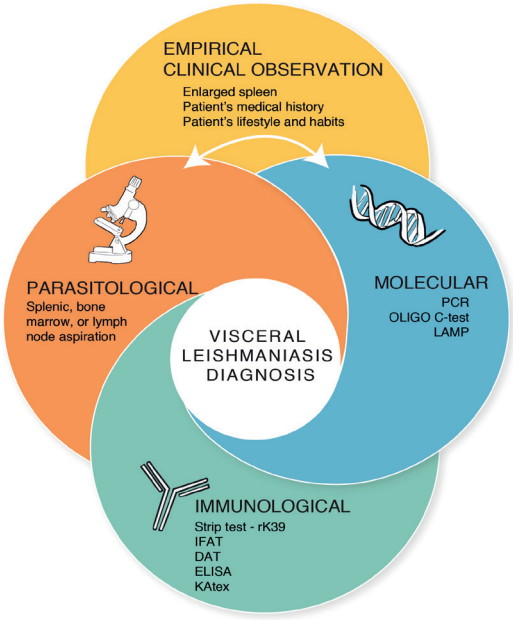

Figure 3.2: Current Methods Used for Diagnosing Visceral Leishmaniasis. After an empirical clinical observation is done, a screening test (usually, an immunological test) is administered, depending upon the availability. Abbreviations: DAT, direct agglutination test; ELISA, enzyme-linked immunosorbent assay; IFAT, immunofluorescent-antibody test; KAtex, latex agglutination test; LAMP, loop-mediated isothermal amplification; PCR, polymerase chain reaction.

3.3.2 Select a Region for a Case Study

Leishmaniases remain endemic in more than 98 countries with the majority of VL cases in South Asia (India, Nepal and Bangladesh), South America (Brazil), and the horn of Africa (Ethiopia, Somalia, South Sudan, Sudan, Kenya and Uganda) (Figure 3.3).[31] Seven countries in particular (India, Brazil, Ethiopia, Kenya, Somalia, South Sudan, and Sudan), reported approximately 90% of the global cases of VL in 2015.[29] Although various VL control programs that focus on prevention and treatment are operational within these countries,[36] the current rK39 RDT has been shown to have a poor performance in East Africa compared with India,[29,37] necessitating an improved diagnostic approach in East African countries.

Considering the global spread of VL, our first challenge was to select a VL endemic region that was conducive for field research. Based on our selection criteria (see Methodology), we selected western Kenya and northeastern Uganda for our field research.

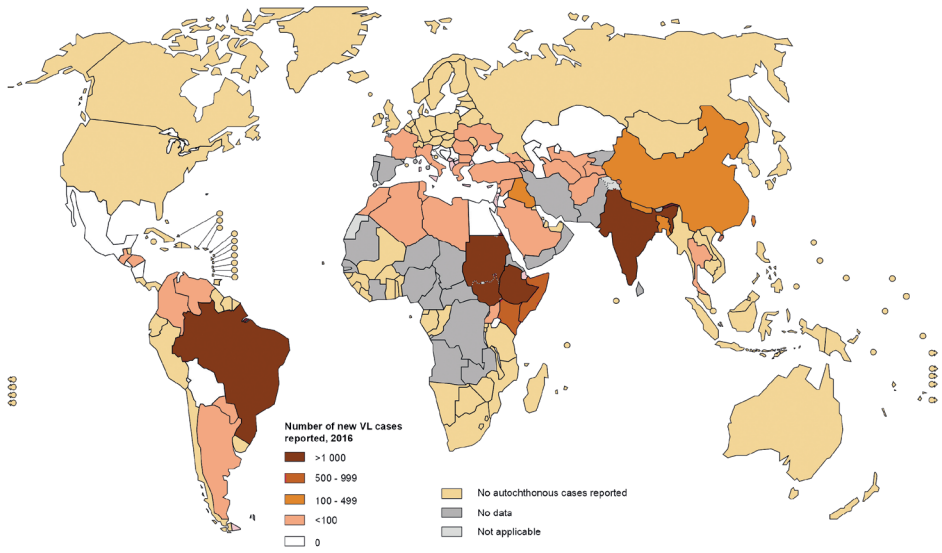

Figure 3.3: Status of Endemicity of Visceral Leishmaniasis Worldwide. Image Source[31].

3.3.3 Field Research With Direct Observations and Interviews With Stakeholders

During our field trip to western Kenya and northeastern Uganda, we visited several Pokot tribal communities, health care facilities, and local organizations over 2 weeks in November 2018 (Figure 3.4). Our international team consisted of scientific researchers including a principal investigator, a postdoctoral researcher, and a PhD researcher, who are working together to develop innovative POC diagnostic tests for infectious diseases, and an industrial-design master student. Our local Kenyan team consisted of a public health officer, a research technician, and a research assistant. We engaged with county and subcounty officials, as well as with local health administrators and community health volunteers (CHVs). Our Ugandan team consisted of a medical doctor and a community health worker (CHW), and we engaged with key stakeholders such as the local chief in the Moroto district. A significant number of interviews were conducted as we followed the recommendations and advice of the locals about whom to speak to (Figure 3.4).

In Kenya, VL testing and treatment can be accessed at the local health care facilities that are located in the Rift Valley region: the Kimalel health center and a newly constructed treatment facility at the Chemolingot subcounty hospital (both within the Baringo County), and the Kacheliba health center (within the West Pokot County). In Uganda, the Amudat hospital is the only VL treatment facility. VL testing can be accessed in Rupa subcounty and by a mobile CHW in the surrounding regions, and patients are referred to the Amudat hospital for further testing and treatment.

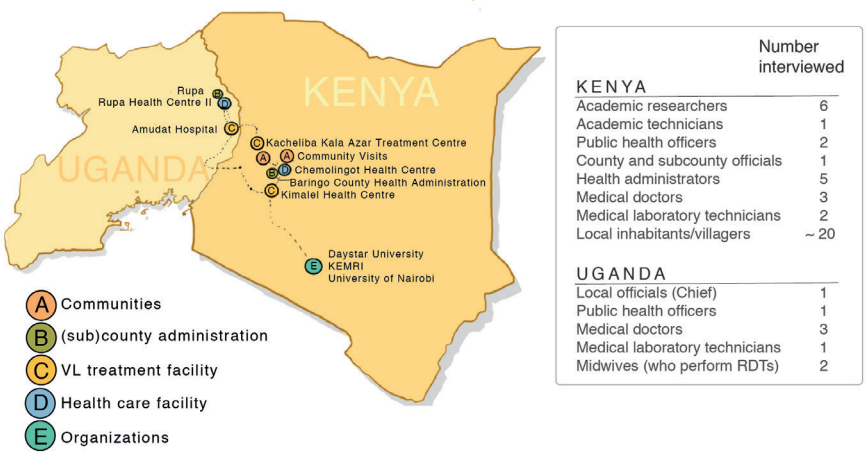

Figure 3.4: Number of Interviews Conducted with Stakeholders on Diagnosing VL in Western Kenya and Northeastern Uganda. RDT, rapid diagnostic text; VL, visceral leishmaniasis.

3.3.4 Create Gigamaps and Patient Journeys Based on Insights Gathered

A Gigamap of the health care system in western Kenya and northeastern Uganda was created based on the literature as well as on the insights obtained in the field. Seven phases were identified (Figure 3.5), from exposure to vectors at home (phase 1); being sick (passive) (phase 2); seeking care (active) (phase 3); getting diagnosed with VL (phase 4); getting to a treatment facility (phase 5); getting treatment (phase 6); and finally, to being treated and going home (phase 7). The Gigamap visualized the journey through the different levels of the health care system, starting from the rural setting (close to homesteads) and advancing towards more urban settings for treatment. The Gigamap also visualized the multiple stakeholders that are involved in the VL health care system.

Next, we created patient journeys (Figure 3.6). We related these journeys to the 7 phases defined in the Gigamap, to obtain a detailed overview of the challenges faced by a patient while seeking effective diagnoses and subsequent treatment. The 8 patient journeys (numbered as I to VIII) were a result of the information gathered in the field, and they represent a sequence of interactions between the patient, the health care system, and the stakeholders involved. A detailed description of patient story II can be found in the supplement. Possible factors and barriers encountered during the patient journeys were identified. Several factors determined the progress of a patient through these different phases resulting in the least efficient (story II) and the most efficient (story VIII) journey from infection to the treatment.

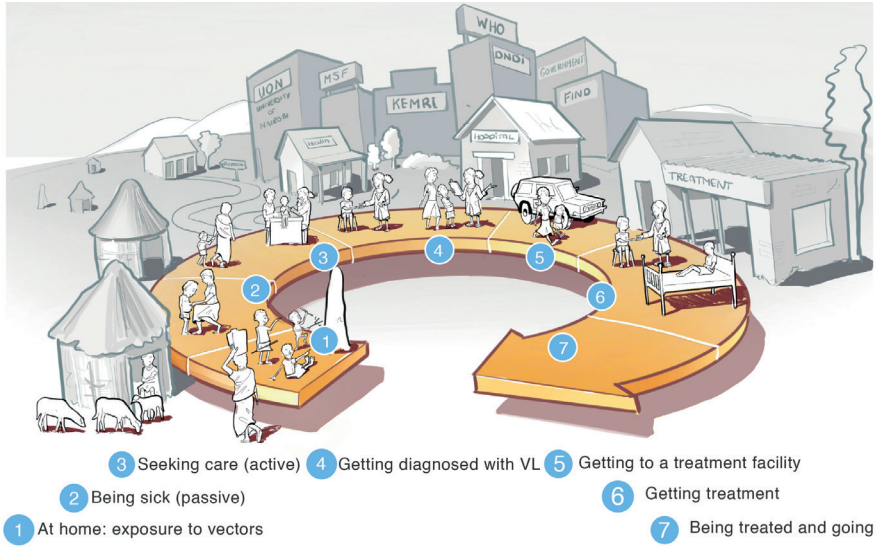

Figure 3.5: Gigamap Diagram Showing the Journey of a Patient Who Has Visceral Leishmaniasis from Infection to Treatment in Western Kenya and Northeastern Uganda.

An important observation during our study was the key role of the CHV/CHW as the closest link to the people in rural communities. In Kenya, CHVs and CHWs are trained on a variety of health issues including case definition, prevention, and control of common ailments, as well as nutrition and family planning. They facilitate access to health services through advocacy, outreach, referral, community education, informal mentoring, and social support.[39] Thus, their role is to identify patients at the homesteads (small clusters of homes) (Figure 3.6, phases 1 and 2) and refer them to the local health care facilities for further diagnoses (Figure 3.6, phase 4). In Uganda, we observed that CHWs were trained to perform VL diagnostics in the field using the rK39 RDT. Due to ongoing VL clinical trials, which raises funding availability, CHWs in Uganda also had access to a motorcycle to allow them to reach secluded homesteads.

In contrast, due to limited clinical trials and a subsequent lack of funding, we observed that CHVs in Kenya are involved in identification of patients and referring them to local health care facilities. CHVs are well-respected members of the communities, such as teachers and ministers, and are trusted by the locals. Furthermore, due to language barriers between the locals and researchers from abroad, CHWs and CHVs played a key role in most interactions with the locals, patients, and health professionals.

The distance to a health care facility for diagnoses (Figure 3.6, phase 4) and treatment (Figure 3.6, phase 6) was found to be the most significant barrier that patients face when seeking health care. Diagnosis may take place at the homesteads by a CHW/CHV or at a health care facility, while treatment would take place at a higher-level facility such as a local hospital. Once diagnosed, either at the homesteads by a CHW/CHV or at a health care facility, patients in the East Pokot subcounty in Kenya that may have VL based on the rK39 RDT, currently travel approximately 80 km to the Kimalel hospital for another (confirmation) diagnostic test and for treatment, if needed. Due to the toxicity and costs of the treatment, patients are diagnosed multiple times to ensure that treatment is only prescribed when absolutely necessary (in contrast to antimalarial treatment that is prescribed more readily). The Chemolingot subcounty hospital treatment facility in Kenya is currently improving accessibility to treatment for patients in the East Pokot subcounty. However, in Uganda, patients from across the country need to travel to Amudat for VL treatment. Interestingly, many Turkanas in Kenya travel approximately 100 km to the Amudat hospital to seek treatment due to conflicts between their tribe and the Pokot in Kenya.

Overlapping symptoms with other febrile illnesses often lead to misdiagnoses based on empirical clinical observations (signs and symptoms) (Figure 3.6, phases 2–7). In VL endemic regions, acute fever is often associated with malaria and other

prevalent tropical diseases. Malaria RDTs and treatment are generally readily available, which encourages their use. Furthermore, a lack of VL awareness, even at the health care centers, promotes such misdiagnoses. Health education influences the ability of patients and health care workers to recognize VL. Increased VL awareness will have a positive influence on the journey of the VL patient because it increases the likelihood of recognizing VL at an early stage. Furthermore, we learned that children play in the termite mounds that are preferred resting and breeding sites for sandflies (the VL vector), and men sleep outside at night, which further exposes them to sandfly bites. Important risk factors include area ecology (humidity, heat), vegetation (acacia trees), livelihoods (pastoralism and proximity to livestock), and general behavior (outdoor sleeping). A significant lack of resources is apparent for the diagnosis and treatment of VL, particularly in terms of health care personnel, diagnostic testing, and financial resources. Despite traveling far distances, patients have no guarantee that diagnostic procedures will be practiced immediately once they arrive at a health care facility for diagnoses. We learned that technicians generally close their laboratories for a day or two when they need to be in the field or are receiving training.

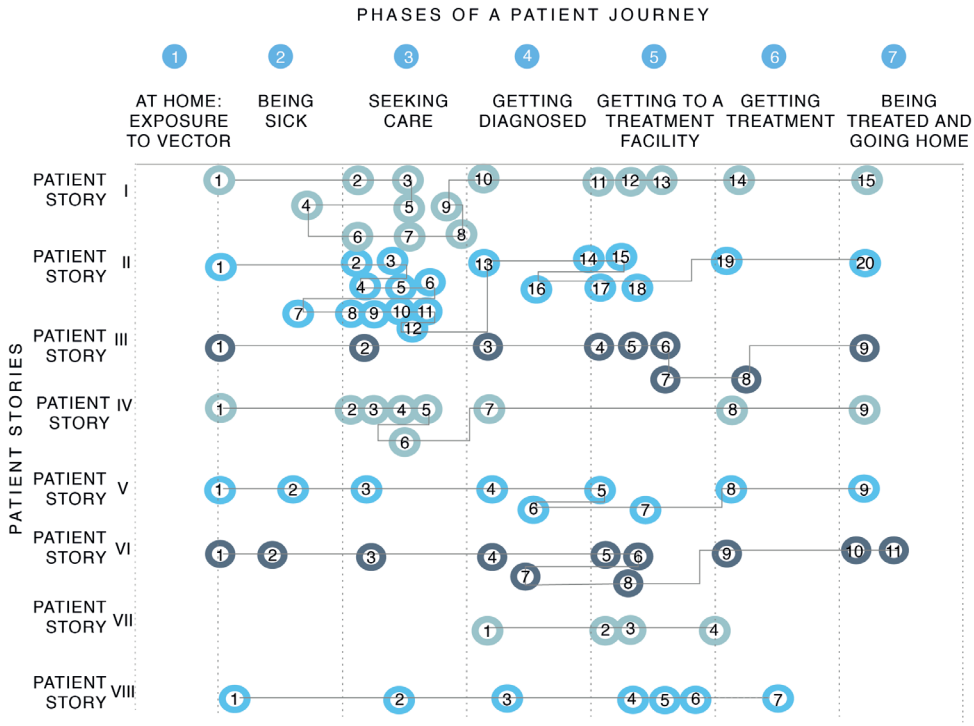

Figure 3.6: Phases of Patient Journeys That Represent the Number of Steps That Patients With Visceral Leishmaniasis Take From Infection to Treatment.

Due to a general shortage of staff, diagnostic procedures are often delayed. Furthermore, RDTs such as the rK39 for VL are not always available, which promotes misdiagnosis of VL. Midwives at the Rupa health center in the Moroto district in Uganda, who routinely perform RDTs for malaria and other illnesses, informed us that the supply of RDTs for VL is not consistent. Thus, symptomatic patients that do not respond to malaria medication are then referred to the Amudat hospital (120 km) for diagnoses and treatment. Finally, the cost of traveling to a health care facility is a significant barrier, particularly when patients are asymptomatic and/or are simply not convinced that they are sick due to a lack of awareness. In general, a lack of financial resources contributes to poor health-seeking behavior (Figure 3.6, phase 2).

We learned that many VL patients know that they are sick (Figure 3.6, phase 2), but do not actively seek health care (testing and treatment) due to the inaccessibility of health care facilities and the fear of encountering other tribes that may be hostile. We also learned that the Pokot and Turkana tribes in Kenya and Uganda are often in conflict, which makes it unsafe for members of either tribe to travel freely to seek health care. Such tribal conflicts inhibit patients from seeking health care and force them to travel to more distant health care facilities that are located away from a conflict zone. Such delays in seeking health care could further worsen the patient's health, and they are often severely anemic and weak by the time they reach a health care facility. Furthermore, patients require screening for multiple infections, including VL, malaria, and tuberculosis, which is challenging when the patients are extremely weak. Stabilizing patients before starting them on VL treatment is also challenging because they may require blood transfusions and treatment for comorbidities, such as malaria, which require urgent attention before they can start VL treatment.

We observed that in general, VL patients also seek care from a traditional healer before considering visiting a health care facility because traditional healers are trusted, located closer to the homesteads, and often alleviate some of the initial symptoms. These deferrals worsen the patient's health, thereby making the treatment more difficult. Unfortunately, critically ill patients often do not respond to the VL treatment, resulting in death. Such cases further strengthen the traditional beliefs and set a negative impression of health care facilities in the minds of health care seekers. Due to financial constraints and the cultural beliefs of the local population,[40] the health care facility usually needs to arrange the last rites because the family does not come to the hospital to take responsibility for the deceased. We observed a traditional patriarchal society, in which males leave the homesteads for work, and females take care of the homes and the children. For males, the loss of income due to the time that is spent traveling to seek health care contributes to poor health-seeking behavior (Figure 3.6, phase 2). For females, being unable to leave children unattended at home contributes to poor health-seeking behavior.

We learned that women from the Pokot tribes often need permission from their husbands to leave their homes before taking themselves or a child to a health care facility. Thus, unequal decision-making power in the household contributes further toward delayed diagnoses and VL treatment for women and children.

Given all these factors, the total number of steps a patient takes to seek treatment can vary significantly. For example, seeking care in phase 3 requires many steps in complicated patient journeys, such as for patient stories I and II (Figure 3.6). This may be due to several of the aforementioned factors. For example, a lack of resources in the unavailability of staff or RDTs may result in the patient returning home (Figure 3.6, phase 2) without receiving a diagnosis, and later traveling back to seek care (Figure 3.6, phase 3). Traveling between phases 1 and 2 may be further hindered by other factors such as distance between the homesteads and the health care facilities or a lack of financial resources. Conversely, a patient journey may be as simple as patient story III whereby a patient progresses easily from being sick (Figure 3.6, phase 2) to being treated and going home (Figure 3.6, phase 7).

3.3.5 Create Use Case Scenarios

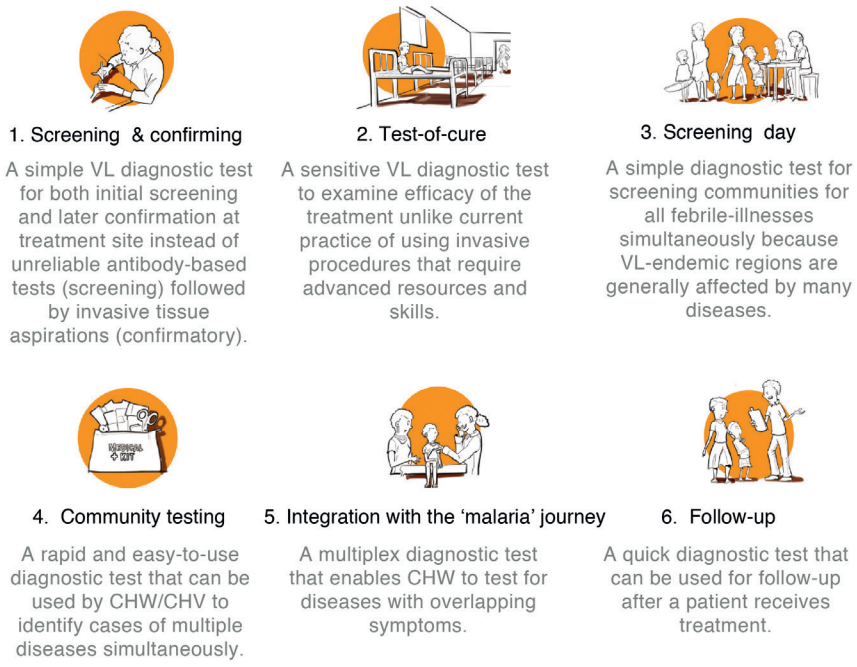

Figure 3.7: Six Scenarios That Elaborate on the Scope of Application of a New Point-of-Care Diagnostic Test. CHV, community health volunteer; CHW, community health worker; VL, visceral leishmaniasis.

Prompted by our extensive methodology, whereby we mapped the health care system in a specific endemic region and analyzed patient journeys, we sketched 6 use case scenarios based on the scope of application of a new POC diagnostic test (Figure 3.7). These different scenarios are based on considerations involving 3 elements: the characteristics of the new diagnostic technology, the contextual fit, and the local need. Each of the scenarios describes and visualizes a potential specific health care context in which a test based on a new diagnostic technology could be of added value to improve diagnostics in the health care system. Thus, each scenario represents a potential diagnostic setting.

3.3.6 Validation of the Use Case Scenarios

The aforementioned 6 scenarios were discussed with experts in the field, including a Kenyan public health officer and VL specialists at Médecins Sans Frontières, to identify the most urgent need based on the perspective of health care providers. Thereafter, we chose a researcher-centric approach to obtain a context-specific diagnostic need that would facilitate researchers to outline the technological requirements of a new POC test. Interestingly, 2 scenarios—screening and confirmation and a test-of-cure—were consistently identified as a priority for developing a CTPP, and we did not obtain any discrepancies in the opinions of the experts. The selection of these 2 scenarios was based on the following criteria: how well the scenario represents a diagnostic setting in the current health care context of VL; how well the scenario meets a local need in terms of VL case management; and how feasible it would be to implement a new POC test in the scenario.

The first scenario selected, “screening and confirmation,” is beneficial for a number of reasons. Given the poor performance of the serological rK39 RDT in east African countries, a more specific and more sensitive POC diagnostic test that can be implemented by end users with minimal training at the lowest level of the health care system is clearly required. Furthermore, it is crucial to consider the end user(s) of a POC diagnostic test because the level of training and availability of resources will influence the diagnostic setting. Thus, a simple, noninvasive, yet effective VL POC diagnostic test that can be used for initial screening and confirmation (scenario 1) would significantly improve VL case management because it would replace the rK39 RDT for initial screening and invasive splenic aspirations for confirmation. The added value of an effective screening and confirmation test is that patients could be screened more reliably at the lowest level of the health care system, for example, by a CHV/CHW who is closer to the homesteads, which would prevent patients from traveling unnecessarily to regional health care facilities.

The second scenario, “test-of-cure,” is useful because it would replace cumbersome procedures (i.e., microscopic analysis of invasively obtained splenic aspirations or

molecular tests such as polymerase chain reaction) that are currently used for test-of-cure. Serological tests cannot serve as test-of-cure owing to persisting antibodies after VL treatment. Thus, a POC diagnostic test that can serve as a test-of-cure (scenario 2) would significantly improve VL case management by replacing invasively obtained splenic aspirations. The added value of a test-of-cure is that relapse of the disease, which occurs in approximately 10% of VL patients, could be detected at an early stage after treatment. Therefore, the test-of-cure scenario fills a critical gap in VL case management.

Scenarios 3 to 6 were excluded from further development of a CTPP. A screening day, as depicted in scenario 3, would be challenging to implement because one cannot ethically test everyone in a community, especially not asymptomatic patients, while symptomatic patients are covered in scenario 1. Additionally, it is difficult to get people to travel to a central location for a screening day because financial constraints or daily routines of herding cattle or taking care of the homesteads often restrict travel. Community testing, as depicted in scenario 4, is dependent on a multiplexed POC test that tests for multiple diseases simultaneously. Multiplexed tests are often more expensive and very few, if any, have been routinely used in the field.

Similarly, integration with the malaria journey, as depicted in scenario 5, is also dependent on a multiplexed POC test for VL and malaria. A well-known multiplex test from DIAMED for VL and malaria is relatively expensive and not routinely used in Kenya and Uganda. By contrast, malaria RDTs are affordable and are routinely used in the field. A follow-up test, as depicted in scenario 6, would be challenging to implement due to the nomadic nature of the inhabitants that we encountered.
A unidirectional problem-solving approach, instead of an interactive design-thinking approach that requires multiple iterations, would have yielded possibly 1 or 2 predictable scenarios instead of the detailed thorough 8 scenarios that were obtained in this study. Thus, after multiple iterations with a Kenyan public health officer and Médecins Sans Frontières, we concluded that scenarios 1 and 2, a test for screening and confirmation and a test-of-cure, are pivotal for VL management as they meet the local need and are feasible to implement. Furthermore, the CTPP developed for the 2 selected scenarios could be broadly applicable to the other 4 scenarios.

3.3.7 Define the CTPP

Within each of these 2 selected scenarios, we identified variables that clarify how the diagnostic setting influences the features of the diagnostic test. A CTPP such as formulated in Figure 3.8 presents the key features of a product that would fit a particular local health care context. The key features that a CTPP takes into account include variables such as why a test is performed (scope); where the test will take place (geographical location); which level of the health care system (such as at a

home or a hospital); who the end user is (target user of the test); when the test will take place in the patients' health-seeking pathway (diagnostic moment); and how the test will be conducted (operational characteristics of the test). In this study, the product is a new POC diagnostic test for VL in rural Kenya and Uganda. A CTPP is an efficient mapping tool that can guide the R&D of a new POC diagnostic test by determining the local needs the end user(s), the intended use, and the adequate features of the test. We conclude that the approach presented in this study led to the development of a CTPP wherein the essential features of a POC diagnostic test for VL in Kenya and Uganda were identified more quickly compared with a TPP.

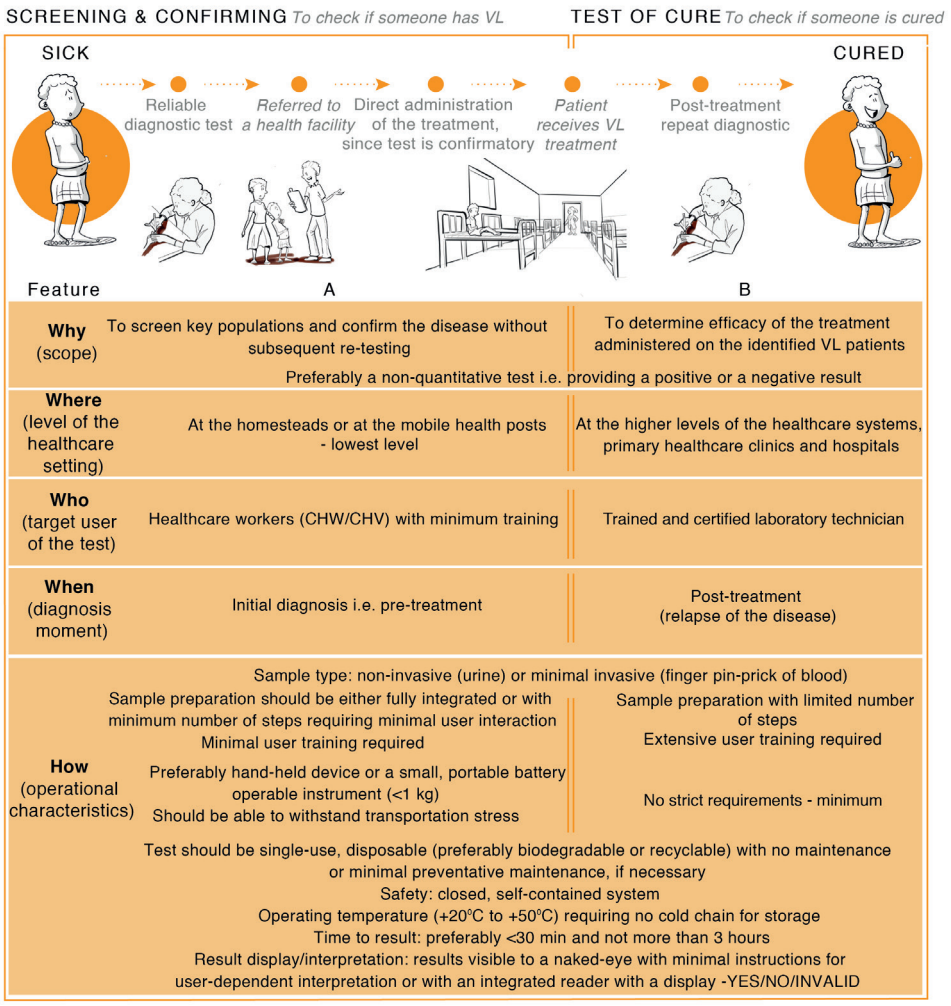

Figure 3.8: (A) CTPP for a VL Point-of-Care Diagnostic Screening-and-Confirmation Test, (B) CTPP for a VL Point-of-Care Diagnostic Test of Cure. CHV, community health volunteer; CHW, community health worker; CTPP, concept target product profile; VL, visceral leishmaniasis.

The added value of a CTPP is that it does not impose overly stringent guidelines on the researcher during the early stages of R&D. Instead, a CTPP aims to guide the research and not limit the potential of the new POC diagnostic test that is under development.

3.4 Discussion

The global diagnostic need for NTDs demands innovative solutions that are customized to the local health care context. To develop a technology that can be implemented in the relevant context, researchers must get access to a comprehensive, yet easily accessible overview of the status quo to understand the challenges and limitations of the existing health care system. Despite extensive literature reviews on VL diagnostics, treatment, and management, an evident knowledge gap persists, which prompted us to conduct field research in VL endemic regions and develop the notion of a CTPP.

Although it is imperative to conduct a comprehensive context analysis before and during R&D of the technology, this is often done only at a much later stage by specialized teams, which may not include scientific researchers. The present study was conducted by scientific researchers who are developing a POC diagnostic test in collaboration with industrial-design experts to define scientifically feasible innovative solutions for VL diagnosis in a resource-limited setting. In contrast to a TPP that comprises a much larger group of experts such as social scientists and policymakers, a CTPP can be formulated effectively in a fast manner with a team that includes technical researchers, health care experts, and industrial designers.

Initially, it appeared challenging to determine how to effectively engage with local stakeholders during a limited time in the field. From the very beginning of our approach, we overcame many logistical and cultural barriers by engaging with local stakeholders. Apart from engaging with medical professionals (doctors, nurses, and laboratory technicians), we spent valuable time with the CHWs/CHVs and observed their crucial role in VL diagnosis in the field. We observed many similarities in the health care systems between Kenya and Uganda, which is unsurprising given the common border and a shared history; therefore, we created a combined Gigamap of their VL health care systems. We observed many challenges that are encountered by the locals: food and water insecurity, which causes malnutrition; remoteness; and a lack of infrastructure and poor health care systems, which collectively adversely affect access to adequate diagnoses and treatment. Furthermore, the lack of resources, both financial and in the availability of health care staff and RDTs for VL, and poor health-seeking behavior, which is exacerbated by the lack of education that is prevalent in the remote regions, impede effective VL management.

Conducting field research provided rich sources of information for understanding how new POC diagnostic tests can fit into a specific health care context. We would like to highlight the importance of international cocreation through active collaborations between all stakeholders, including academia, industry, nonprofit, and governmental organizations.[9] Cocreation with local experts is necessary to understand the implementation need for a new POC diagnostic test, as well as to ensure the sustainable use of the POC test in the field by building trust and mutual interest and creating a foundation for knowledge transfer to engage locals in the future. New technologies are often mistrusted by health care providers, especially in remote settings, due to a lack of understanding of the complex research behind the development of the new technologies. Thus, cocreation with the local stakeholders starting from the design phase (conceptualization) to the prototype phase (realization) ensures a strong relationship with the end users. The approach that we adopted, in fact, promoted collaboration (and not competition) between the key stakeholders.

A number of key findings that were learned from the approach are as follows:

1. A CTPP is an effective new tool that can aid R&D researchers in matching the technology that they develop to a specific health care context more quickly than a conventional target product profile.

2. The role of local volunteers and community health care workers is critically important for access to diagnostics in resource-limited settings. With improved yet simplified VL POC diagnostic tests, CHWs/CHVs could perform diagnosis of VL closer to the homesteads.

3. A noninvasive test-of-cure and a screening and confirmation test will significantly improve the management of VL in the endemic regions. This would greatly benefit patients, particularly immunocompromised patients who are at a higher risk of relapse, as well as help pharmaceutical researchers and clinicians who are developing and testing new VL treatment regimens.

4. The cost of the diagnostic test is an important factor to consider, especially in view of the fact diagnoses needs to be repeated, to screen the patient in the field initially, and again at the treatment facility to rule out any procedural error and to justify the toxicity and expense of VL treatment. Thus, the diagnostic test needs to be affordable.

Key implications learned from the approach are:

1. Early during the R&D stage, researchers should consider who will administer the test (patient, health care worker, doctor) and for what purpose.

2. Program managers should consider that the training level of staff and

volunteers and the availability of the resources are the critical determinants for using a diagnostic test.

3. Researchers should consider that introducing themselves to local communities and stakeholders early will improve the willingness of the communities to implement the new technology. Upon further development, testing prototype devices can be facilitated by the local East African partners, thus strengthening international cocreation, and increasing the probability of success of a new POC diagnostic test beyond a mere proof-of-principle.

The plethora of information gathered in the field was comprehensively processed using our methodology, which includes design thinking tools such as visual thinking, leading to the development of a Gigamap, patient journeys, and the consequent use case scenarios that are presented in this study. The visual thinking was used as a means to summarize, validate, and communicate key insights from the field research to the stakeholders, as well as to create an aligned vision within the team. The visualizations allowed us to identify where, when, and how a new POC diagnostic test can fit into the health care system within a resource-limited endemic region, in the form of a CTPP, which is an efficient mapping tool compared with a TPP. A CTPP approach was applied to sufficiently scope the problem of VL diagnostics, gather contextual information, and define the adequate features of a new POC diagnostic test.

3.5 Conclusion

Disease eradication requires improved diagnostic tests, as well as an efficient system to successfully deliver and implement them in the appropriate settings.[41] As this is largely dependent on the local capacity and the willingness of key stakeholders to participate, solely designing a POC test for a particular setting does not ensure successful implementation of the test.[5] Designing a product for the end user is complex since a wide range of political, social, cultural, and environmental factors contribute, but it is worth the added time, effort, and resources to realize a successful development and implementation of a new POC diagnostic test.

In this article, we presented an approach that included design-thinking principles to formulate a CTPP that consists of multiple steps. Our approach moved from identifying gaps in current VL diagnosis in endemic regions by critically reviewing the existing literature, to selecting an endemic region to validate the literature findings and conduct direct observations in the field. After that, we used visual thinking to create Gigamaps and patient journeys based on the combined insights that were obtained from the literature and the field research, which led to valuable use case scenarios that describe the ideal setting for a new POC diagnostic test. Finally, we used these collective data to formulate a CTPP for a new POC diagnostic test that is

specific for VL diagnostics in resource-limited settings.

In summary, we introduced the notion of a CTPP as an effective toolbox to match the development of a POC diagnostic test and the health care context for its application. More generally, we anticipate that a CTPP will be a useful new tool that enables researchers to match the development of new diagnostic tests or medical equipment, and the local health care context in which they will be used. We envision that a CTPP will enable researchers to ruminate on the new product and facilitate the iterative design process—and ultimately benefit global health.

3.6 Supplementary Information

Patient story II – A 5-year-old who was referred multiple times

1. A 5-year-old girl does not feel well. She has a fever and general malaise.
2. Her mother assumes that she has malaria and goes to the nearest health care facility to get malaria medication that is readily available.
3. The 5-year-old girl takes the malaria medication but does not feel better.
4. Her mother asks their neighbor for advice but she also does not know what is causing the illness. Weeks pass by.
5. The 5-year-old girl gets even more sick. Her spleen starts to swell. Her mother decides to take her back to the health care facility.
6. At the health care facility, they test the 5-year-old girl for malaria. The test results are negative. However, they are sure it is malaria because her symptoms are the same as malaria symptoms. They prescribe another dose of malaria medication and send the girl home.
7. The 5-year-old girl does not get better. She gets progressively more ill.
8. Her mother takes her back to the health care facility and asks for help because she is convinced now that the 5-year-old girl does not have malaria.
9. The nurse agrees that it cannot be malaria and tests the 5-year-old girl for other illness such as tuberculosis. However, all the test results are negative.
10. The 5-year-old girl is then referred to another health care facility. The mother and child walk 80 km to the next health care facility.
11. At the health care facility, another nurse tests the 5-year-old girl for several illness including malaria. The nurse suspects that the 5-year-old girl might have visceral leishmaniasis (VL) but she does not have any VL tests. The 5-year-old girl is then referred to the nearest hospital.
12. The mother carries the child to the nearest hospital which is 20 km away. This journey takes many hours.
13. The 5-year-old girl is tested positive for VL with the rK39 rapid diagnostic test (RDT). She is referred to another hospital that also has a VL treatment center.
14. The staff call the VL treatment center and request a vehicle to transport the

5-year-old girl—without her mother who has to return home.

15. The vehicle transports the 5-year-old girl to the treatment center.
16. When the 5-year-old girl arrives at the treatment center, she is tested again for VL with the rK39 RDT. The result is again positive. She then gets further blood tests done for liver function and anemia, to name a few. These tests are required before VL treatment will be administered.
17. The blood tests reveal that the 5-year-old girl's hemoglobin levels are too low for treatment (less than 4.0 g/dL).
18. The 5-year-old girl is then taken to another treatment center for a blood transfusion before she can start VL treatment. This takes 1 week.

3.7 Appendix: Photographs that illustrate the local context in Kenya. Photo credit: Astrid ten Bosch and Cees Dekker.

Local homesteads in a very remote region in East Pokot.

Termite mounds are breeding sites for sandflies (vector for visceral leishmaniasis) (top). Termite mounds are found in very close proximity to local homesteads (bottom).

Males sleep outside on cow skin (used as a mattress) (left) and females sleep inside on a bed that is made from branches (right).

Kala-Azar (visceral leishmaniasis) treatment centre (top) and dedicated visceral leishmaniasis treatment ward (bottom).

Laboratory bench (top) and Giemsa staining station for microscopy (bottom).

Central well-equipped laboratory (top) and the TU Delft team engaging with local laboratory experts (bottom).

Members of the TU Delft team (Professor Cees Dekker and Michel Bengtson) engaging with the local communities.

References

1. Li Y, Li S, Wang J, Liu G. CRISPR/Cas systems towards next-generation biosensing. Trends Biotechnol. 2019;37(7):730-743.
2. Florkowski C, Don-Wauchope A, Gimenez N, Rodriguez-Capote K, Wils J, Zemlin A. Point-of-care testing (POCT) and evidence-based laboratory medicine (EBLM)—does it leverage any advantage in clinical decision making? Crit Rev Clin Lab Sci. 2017;54(7-8):471-494.
3. Gootenberg JS, Abudayyeh OO, Kellner MJ, Joung J, Collins JJ, Zhang F. Multiplexed and portable nucleic acid detection platform with Cas13, Cas12a, and Csm6. Science. 2018;360(6387):439-444.
4. Hotez PJ, Pecoul B, Rijal S, et al. Eliminating the neglected tropical diseases: translational science and new technologies. PLoS Negl Trop Dis. 2016;10(3):e0003895.
5. Aranda Jan C, Jagtap S, Moultrie J. Towards a framework for holistic contextual design for low-resource settings. Int J Design 2016;10:3. Accessed August 24, 2020. http://www.ijdesign.org/index.php/IJDesign/article/view/2596.
6. World Health Organization (WHO). Ending the Neglect to Attain the Sustainable Development Goals—a Road Map for Neglected Tropical Diseases 2021–2030. WHO; 2016. Accessed August 24, 2020. https://apps.who.int/iris/bitstream/handle/10665/332094/WHO-UCN-NTD-2020.01-eng.pdf?ua=1.
7. Cruz I, Albertini A, Barbeitas M, et al. Target product profile for a point-of-care diagnostic test for dermal leishmaniases. Parasite Epidemiol Control. 2019;5:e00103.
8. Engel N, Wachter K, Pai M, et al. Addressing the challenges of diagnostics demand and supply: insights from an online global health discussion platform. BMJ Glob Health. 2016;1(4):e000132.
9. Dittrich S, Tadesse BT, Moussy F, et al. Target product profile for a diagnostic assay to differentiate between bacterial and non-bacterial infections and reduce antimicrobial overuse in resource-limited settings: an expert consensus. PLoS One. 2016;11(8):e0161721.
10. Cruz I, Albertini A, Barbeitas M, et al. Target Product Profile for a point-of-care diagnostic test for dermal leishmaniases. Parasite Epidemiol Control. 2019;5:e00103.
11. Ding XC, Ade MP, Baird JK, et al. Defining the next generation of Plasmodium vivax diagnostic tests for control and elimination: target product profiles. PLoS Negl Trop Dis. 2017;11(4):e0005516.
12. PATH. Diagnostics for Neglected Tropical Diseases: Defining the Best Tools Through Target Product Profiles. PATH; 2015. Accessed August 24, 2020. https://path.azureedge.net/media/documents/DIAG_ntd_prod_profile_rpt.pdf.
13. McNerney R. Diagnostics for developing countries. Diagnostics (Basel). 2015;5(2):200-209.
14. Kleinsmann M, Valkenburg R, Sluijs J. Capturing the value of design thinking in different innovation practices. Int J Design. 2017;11(2):24-40. Accessed August 24, 2020. http://www.ijdesign.org/index.php/IJDesign/article/view/2771/780.
15. Hasso Plattner Institute of Design at Stanford University. Design Thinking Bootleg. Accessed July 19, 2020. https://static1.squarespace.com/static/57c6b79629687fde090a0fdd/t/5b19b2f2aa4a99e99b26b6bb/1528410876119/dschool_bootleg_deck_2018_final_sm+%282%29.pdf.
16. Diehl JC, Christiaans H. Product service systems: the future for designers? The changing

role of the industrial designer. Paper presented at: International Design Conference of KSDS and ADADA With Cumulus; October 17-24, 2015; Gwangju, South Korea.
17. Sevaldson B. Systems oriented design: The emergence and development of a designerly approach to address complexity. Paper presented at: DRS//Cumulus 2013, 2nd International Conference for Design Education Researchers; May 14-17, 2013; Oslo, Norway.
18. Sevaldson B. Relating systems thinking and design IV. FormAkademisk - forskningstidsskrift for design og designdidaktikk. 2018;11(4).
19. Sevaldson, B. Giga-mapping: visualisation for complexity and systems thinking in design. Paper presented at: Nordic Design Research Conference 2011; May 29-31, 2011; Helsinki, Finland.
20. Manchaiah VKC, Stephens D, Meredith R. The patient journey of adults with hearing impairment: the patients' views. Clin Otolaryngol. 2011;36(3):227-234.
21. Simonse L, Albayrak A, Starre S. Patient journey method for integrated service design. Design for Health. 2019;3(1):82-97.
22. Olobo-Okao J, Sagaki P. Leishmaniasis in Uganda: historical account and a review of the literature. Pan Afr Med J. 2014;18:16.
23. Okwor I, Uzonna J. Social and economic burden of human leishmaniasis. Am J Trop Med Hyg. 2016;94(3):489-493.
24. Handman E. Leishmaniasis: current status of vaccine development. Clin Microbiol Rev. 2001;14(2):229-243.
25. Picado A, Dash AP, Bhattacharya S, Boelaert M. Vector control interventions for visceral leishmaniasis elimination initiative in South Asia, 2005-2010. Indian J Med Res. 2012;136(1):22-31.
26. Mathers CD, Ezzati M, Lopez AD. Measuring the burden of neglected tropical diseases: the global burden of disease framework. PLoS Negl Trop Dis. 2007;1(2):e114.
27. Aronson N, Herwaldt BL, Libman M, et al. Diagnosis and treatment of leishmaniasis: clinical practice guidelines by the Infectious Diseases Society of America (IDSA) and the American Society of Tropical Medicine and Hygiene (ASTMH). Clin Infect Dis. 2016;63(12):1539-1557.
28. Alvar J, Vélez ID, Bern C, et al; WHO Leishmaniasis Control Team. Leishmaniasis worldwide and global estimates of its incidence. PLoS One. 2012;7(5):e35671.
29. Burza S, Croft SL, Boelaert M. Leishmaniasis. Lancet. 2018;392(10151):951-970.
30. Abeijon C, Alves F, Monnerat S, et al. Development of a multiplexed assay for the detection of Leishmania donovani/Leishmania infantum protein biomarkers in the urine of patients with visceral leishmaniasis. J Clin Microbiol. 2019;57(5):e02076-18.
31. Lindoso JA, Cunha MA, Queiroz IT, Moreira CH. Leishmaniasis-HIV coinfection: current challenges. HIV AIDS (Auckl). 2016;8:147-156.
32. Ferede G, Diro E, Getie S, et al. Visceral leishmaniasis-malaria coinfection and their associated factors in patients attending Metema Hospital, Northwest Ethiopia: suggestion for integrated vector management. Malar Res Treat. 2017;2017:6816913.
33. Shweta, Bhatnagar S, Gupta AK, Murti K, Pandey K. Co-infection of visceral leishmaniasis and pulmonary tuberculosis: a case study. Asian Pac J Trop Dis. 2014;4(1):57-60.
34. Srivastava P, Dayama A, Mehrotra S, Sundar S. Diagnosis of visceral leishmaniasis. Trans R Soc Trop Med Hyg. 2011;105(1):1-6.
35. El-Safi S, Chappuis F, Boelaert M. The challenges of conducting clinical research on neglected tropical diseases in remote endemic areas in Sudan. PLoS Negl Trop Dis.

2016;10(11):e0004736-e0004736.
36. Olobo-Okao J, Sagaki P. Leishmaniasis in Uganda: historical account and a review of the literature. Pan Afr Med J. 2014;18:16.
37. Chappuis F, Mueller Y, Nguimfack A, et al. Diagnostic accuracy of two rK39 antigen-based dipsticks and the formol gel test for rapid diagnosis of visceral leishmaniasis in north-eastern Uganda. J Clin Microbiol. 2005;43(12):5973-5977.
38. van Rij E, Nour BYM, Schallig HDFH. Evaluation of urine as a diagnostic specimen for visceral leishmaniasis in Sudan. J Trop Dis. 2013;1(2):106.
39. World Heath Organization: Status of endemicity of visceral leishmaniasis worldwide, 2018. Accessed August 29, 2020. https://www.who.int/leishmaniasis/burden/GHO_VL_2018.pdf?ua=1.
40. Ministry of Public Health and Sanitation, Republic of Kenya (MOH). Community Health Volunteers (CHVs) Basic Modules Handbook. MOH; 2013. Accessed August 24, 2020. http://guidelines.health.go.ke:8000/media/CHV_handbook_PDF-F.pdf.
41. O'Dempsey TJD. Traditional belief and practice among the Pokot people of Kenya with particular reference to mother and child health: 1. The Pokot people and their environment. Ann Trop Paediatr. 1988;8(2):49-60.
42. Hotez PJ, Pecoul B, Rijal S, et al. Eliminating the neglected tropical diseases: translational science and new technologies. PLoS Negl Trop Dis. 2016;10(3):e0003895.

4

CRISPR-dCas9 based detection scheme for diagnostics in resource-limited settings

Nucleic-acid detection is crucial for research and medicine. For effective diagnostics in resource-limited settings, however, most detection schemes are inapplicable since they rely on expensive machinery and trained personnel. Here, we present a novel isothermal DNA detection scheme for the diagnosis of pathogenic DNA in resource-limited settings. DNA was extracted with a pH based chitosan-mediated approach, and amplified using Recombinase Polymerase Amplification with a sensitivity of <10 copies of DNA in a broad temperature range of 25 – 45ºC within 15 minutes. Target DNA was bound by dCas9/sgRNA that was labelled with a DNA oligomer to induce Rolling Circle Amplification, which can be conducted from 15 – 60 ºC. This second amplification step produced many copies of a G quadruplex DNA structure that facilitates a colorimetric readout that is visible to the naked eye. As an example of the applicability of this scheme, we demonstrate detection of DNA of visceral leishmaniasis, a neglected tropical disease. Given the versatility of the guide-RNA programmability of targets, we envision that this nucleic acid detection scheme can easily be adapted to detect any DNA with minimal means, which facilitates point-of-care diagnostics in resource-limited settings.

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4.1 Introduction

The ability to detect DNA and RNA sequences is key to basic research in multiple branches of science[1,2]. DNA detection is also vital in many biosensing applications, e.g., for clinical diagnostics[1], species-specific identification of infectious agents[3], antimicrobial resistance[4], epidemiology studies[5], forensics (genotyping)[6], biodefense[7], food and water safety[8], plant diseases[9], and environmental monitoring for bacterial, viral or pathogenic contamination[10]. Methods for DNA detection include polymerase chain reaction (PCR), more specifically quantitative PCR (qPCR)[11], molecular hybridization techniques such as microarrays[11] or DNA fluorescence in situ hybridization[12], as well as DNA sequencing using platforms such as next-generation-sequencing[11] or nanopore sequencing[13,14]. Recent additions to this broad spectrum of DNA-detection methods include the use of DNA-binding proteins, such as Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) systems and their associated Cas proteins that have been adapted from bacterial immune systems.

One major area of application of nucleic-acid (NA) detection is in medicine. Effective treatment and prevention of infectious diseases requires effective diagnostics[15,16]. Diagnostic methods are either parasitological (microscopy and culture), immunological (antigen/antibody), or molecular (DNA detection)[17]. While PCR is a remarkable DNA detection tool, its use for molecular diagnosis is severely hampered in resource-limited settings as it relies on expensive instruments (thermal cycler), expert personnel to operate it, and a stable source of electricity[18,19,20] – all modest demands that nevertheless are lacking in many endemic regions[21] where infectious diseases like neglected tropical diseases (NTDs) thrive. As such requirements are often not met in resource-limited settings, there is a need for new diagnostic tools that enable minimally trained users to probe for diseases with minimal handling and resources[22], in particular for point-of-care (PoC) diagnostic tests (tests that work at the time and place of patient care[23]) that decentralize diagnosis and mitigate the need for well-equipped central laboratories[22]. The nature of a diagnostic test has implications for its usefulness. Serological tests that rely on the detection of antibodies from the infected person are valuable screening tools, but their efficacy differs between individuals and countries, due to inherently different immunological responses[24]. Serological tests furthermore cannot be used to test the efficacy of treatment (test-of-cure) or re-infection (relapse), due to persisting antibodies after treatment[25,26]. Serological methods such as enzyme-linked immunoassay (ELISA) are rapid, but have high false positive rates and poor stability at room temperature. Conventional culture-based diagnostic methods are accurate, but are laborious and have a slow turnaround time for results (~1 week). Hence, rapid confirmatory diagnostic tests are urgently needed[27]. Direct DNA detection offers many advantages over serological detection[28] as it is independent of the patient's immune system,

it can potentially serve as a test-of-cure[25,26], it is more sensitive and specific than serological methods[3], and more rapid than culture-based methods[29].

A promising avenue for DNA-based PoC diagnostics is the use of programmable nucleic-acid-binding protein systems such as CRISPR-Cas. Notable examples of CRISPR-Cas-based PoC diagnostic platforms include CRISDA[30], Cas9 detection for Zika[31], DETECTR[32], and SHERLOCK[33]. Although SHERLOCK and DETECTR have achieved impressive attomolar sensitivities[34], they still require sophisticated laboratory equipment as several of the handling steps are restricted to multiple incubations at different temperatures. One of the greatest challenges for PoC diagnosis in resource-limiting settings is sample preparation[35,36], and the use of PCR and sequencing technologies for diagnostics in resource-limited settings is restricted by the complex sample preparation[37]. The SHERLOCK DNA detection platform[33], for example, has been coupled to a sample preparation technique known as HUDSON, which relies on heating the samples to 50°C to inactivate nucleases, and to 90°C to inactivate viruses, before detection by CRISPR-Cas13a[36] can be initiated.

To achieve adequate sensitivity in PoC diagnostics, all CRISPR-Cas-based diagnostic platforms rely on isothermal amplification to achieve attomolar sensitivity[34]. Loop-mediated isothermal amplification (LAMP) which operates at a constant but elevated temperature of 65ºC, is extensively used for such PoC diagnostics[34,29,19,38,39]. Many isothermal amplification reactions require an initial heating step to unwind the double stranded DNA (dsDNA) targets which limits their use in resource-limited settings[30]. A prominent example of an isothermal amplification technique that does not require initial heating is recombinase polymerase amplification (RPA), which has been developed into commercially available products[40], but has so far not been widely applied in PoC diagnostics applications.

Here, we present an isothermal DNA-detection scheme that detects pathogenic DNA in human samples and provides a colorimetric readout that is visible to the naked eye (Figure 4.1). We combine this DNA-detection scheme with an instrument-free DNA-extraction procedure[41,42] whereupon the extracted DNA is isothermally amplified by RPA. The amplified DNA serves as a template for detection via CRISPR-dCas9 recognition, using the dCas9 mutant that specifically binds but does not cleave dsDNA. The RPA reaction is performed with a biotinylated primer, which facilitates the binding of amplified DNA to streptavidin beads in the tube. To couple the DNA detection to a colorimetric readout, we attached a DNA oligonucleotide to the dCas9 which hybridizes to a single stranded DNA (ssDNA) circular template for a subsequent rolling circle amplification (RCA) reaction. Upon specific recognition of the target DNA in the sample by the CRISPR-Cas-oligonucleotide complex, this circular RCA template primes a subsequent RCA reaction. The circular RCA template encodes enzymatic G-quadruplexes in tandem repeats that produce the final

colorimetric readout. Below, we describe the successful operation of all these steps, and we specify the favorable sensitivity and temperature ranges where the method operates – demonstrating its potential for PoC diagnostics in resource-limited settings.

For proof-of-principle, we demonstrate this novel DNA detection scheme for the detection of the NTD leishmaniasis[43]. Visceral leishmaniasis (VL), also known as Kala-azar, affects the visceral organs (liver, spleen and lymph nodes) and is curable, but it persists as a fatal disease as it is often left undiagnosed and untreated[44]. Current VL rapid diagnostic tests are serological and sub-optimal[45], indicating a need for PoC development for DNA-based diagnostics in resource-limited settings. To diagnose VL using our novel DNA detection scheme, a highly conserved multicopy region (~10,000 copies/parasite) was identified in the *Leishmania* kinetoplast minicircle DNA, and its presence was validated within a patient's blood and urine sample. We demonstrate the presence of VL DNA with a colorimetric readout that is visible to the naked eye. We anticipate that this DNA detection scheme is broadly applicable for many other diseases and a wider range of biosensing applications.

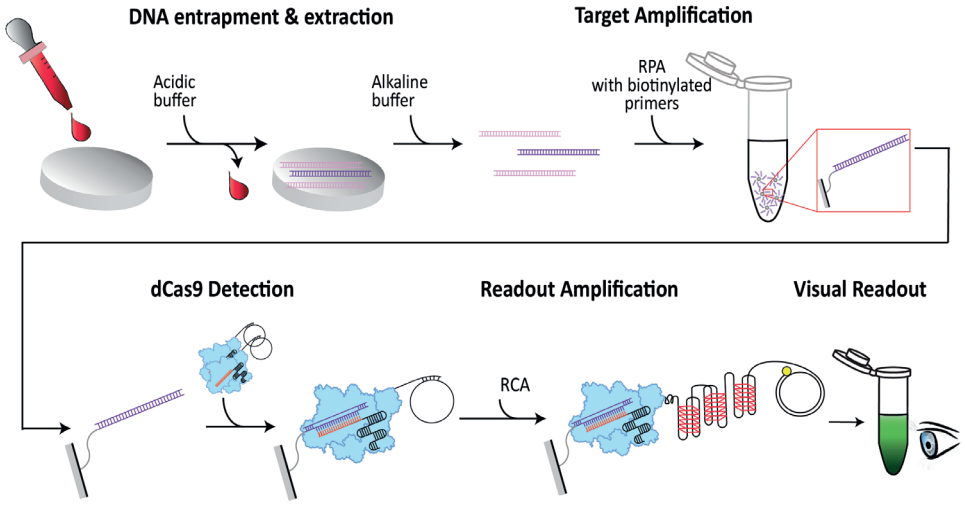

Figure 4.1: Schematic of the DNA detection. DNA extraction: A biological sample (acidic) is administered onto chitosan-functionalized paper discs, resulting in entrapping of the DNA, which is subsequently released upon washing with an alkaline buffer. Target Amplification: Target DNA (purple) is isothermally amplified using RPA with a biotinylated primer in a background of genomic DNA (pink). Subsequently, it is immobilized to streptavidin-coated beads via biotin-streptavidin interactions. dCas9 detection: The RPA-amplified immobilized target DNA is recognized by CRISPR-dCas9 (light blue) that has a single-stranded DNA (ssDNA) circle attached. Subsequently, anything that is not bound to the beads is washed away. Readout amplification: The circular ssDNA is used to prime an RCA reaction. The resulting RCA product consists of tandem repeats of G-quadruplexes. Each of these G quadruplexes picks up a heme group and a colorimetric readout is produced where a visible colour appears in the tube when the target DNA sequence is present.

4.2 Results

4.2.1 Target DNA in biological samples can be detected sensitively, fast, and across a wide range of temperatures

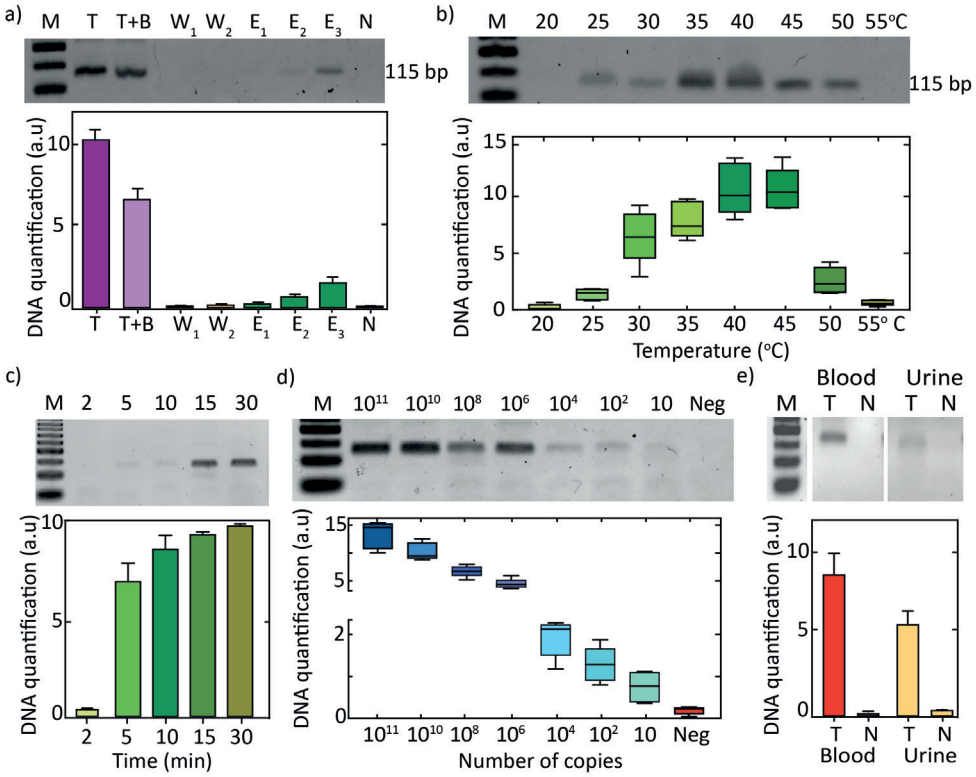

Figure 4.2: chitosan-mediated DNA extraction and subsequent isothermal DNA amplification. a) pH-based chitosan-mediated DNA extraction. Lane 1 (M): 50 bp DNA ladder showing 50, 100, 150 bp oligomers. Lane 2-9 show the 115 bp DNA band. Lane 2 (target, T): PCR-purified target (1012 copies). Lane 3: PCR-purified target in MES buffer, T+B (pH 5.0, 10 µl) that was added onto a chitosan functionalized membrane. Lanes 4 and 5 (W1, W2): result of two separate washes with MES buffer (pH 5.0, 20 µl each) to remove unbound DNA. Lanes 6 - 8 (Elutes 1-3): 3 subsequent elution steps with Tris buffer (pH 8.0, 20 µl each) to extract all bound DNA. Lane 9 (neg): no-target control, i.e., MES buffer (pH 5.0, 10 µl) that was added onto chitosan-functionalized membrane and eluted with Tris buffer (pH 8.0, 20 µl). b) Effect of the operating temperature on the RPA reaction, where DNA from the third elution step (E3 from Figure 2a) was followed by isothermal amplification (RPA) for 30 min at different temperatures from 20 to 55°C. c) Effect of the duration of the RPA reaction, where elute 3 from Figure 2a was followed by RPA for different durations, i.e., 2 - 30 min at 39°C. d) Sensitivity of the RPA reaction: RPA reaction results for different input concentrations of target DNA, ranging from 1011 to 10 copies, that were added unto the chitosan-functionalized membrane for DNA extraction followed by RPA at 39°C for 30 min. e) Chitosan-mediated DNA extraction and subsequent RPA at 39°C for 30 min for biological samples, i.e., blood and urine samples spiked with target (2x1011 molecules). T denotes sample spiked with target DNA, and N denotes negative control. All gels represent a single experiment, while data in the bottom quantifications are plotted for n ≥ 3.

The first steps in our isothermal DNA-detection scheme (Figure 4.1) involve the extraction and amplification of target DNA. To isolate DNA from biological samples, we utilize a pH-based chitosan-mediated DNA-extraction procedure[46] wherein, in acidic conditions, DNA is electrostatically adsorbed onto chitosan-functionalized paper discs (Supplementary Figure 4.1), and subsequently the DNA is eluted with an alkaline buffer wash. To test this, we added target DNA to an acidic buffer (MES buffer, pH 5), or a biological liquid (blood or urine, adjusted to pH 5). Fluid (buffer, blood, or urine) was spiked with target DNA and administered onto chitosan-functionalized paper discs, washed with the acidic buffer (MES buffer, pH 5) to remove unbound constituents, and subsequently washed with alkaline buffer (Tris buffer, pH 8) to elute the DNA, and the results were analysed using gel electrophoresis. As can be seen from Figure 4.2a, DNA was successfully bound to the chitosan-functionalized membrane. DNA was however eluted with three subsequent washes with an alkaline buffer (Tris pH 8.0).

The eluted DNA was used as a template for a downstream RPA reaction. The rehydration buffer from a commercial RPA kit was used as the alkaline buffer for the elution of the DNA that was adsorbed onto the chitosan-functionalized paper discs. The DNA extraction and the subsequent isothermal-amplification steps were functional in a broad temperature range from 25℃ to 50℃ (Figure 4.2b), which is important for applications in PoC diagnostics in resource-limited regions. The assay is also quick, producing a sizeable reading already after 5 minutes (Figure 4.2c). Furthermore, the sensitivity is excellent, as the assay can identify (Figure 4.2d) as few as 10 target DNA copies in the volume corresponding to a blood prick (10 µl). The pH-based chitosan-mediated DNA-extraction approach followed by the RPA reaction exhibited the same detection limit as that of the RPA reaction alone (Supplementary Figure 4.2), indicating that the reactions were compatible and that the chitosan approach did not hinder the amplification efficiency.

Next, the assay was tested with urine and blood samples that were spiked with target DNA ($2x10^{11}$ VL target molecules) (Figure 4.2e). The chitosan-mediated DNA-extraction procedure was found to work very well directly from crude blood and urine samples. Biotinylated primers were used for the RPA reaction to facilitate immobilization of the amplified target DNA onto the streptavidin-coated beads (Figure 4.1, top right), thus allowing to wash off all unwanted reagents. This wash step resulted in a clean amplified target DNA for the subsequent dCas9-based recognition. Notably, the primers could either be immobilized to the streptavidin-coated beads before or during the RPA reaction (Supplementary Figure 4.3).

4.2.2 CRISPR-dCas9 on target DNA can be bound, amplified, and visualized with a colorimetric readout

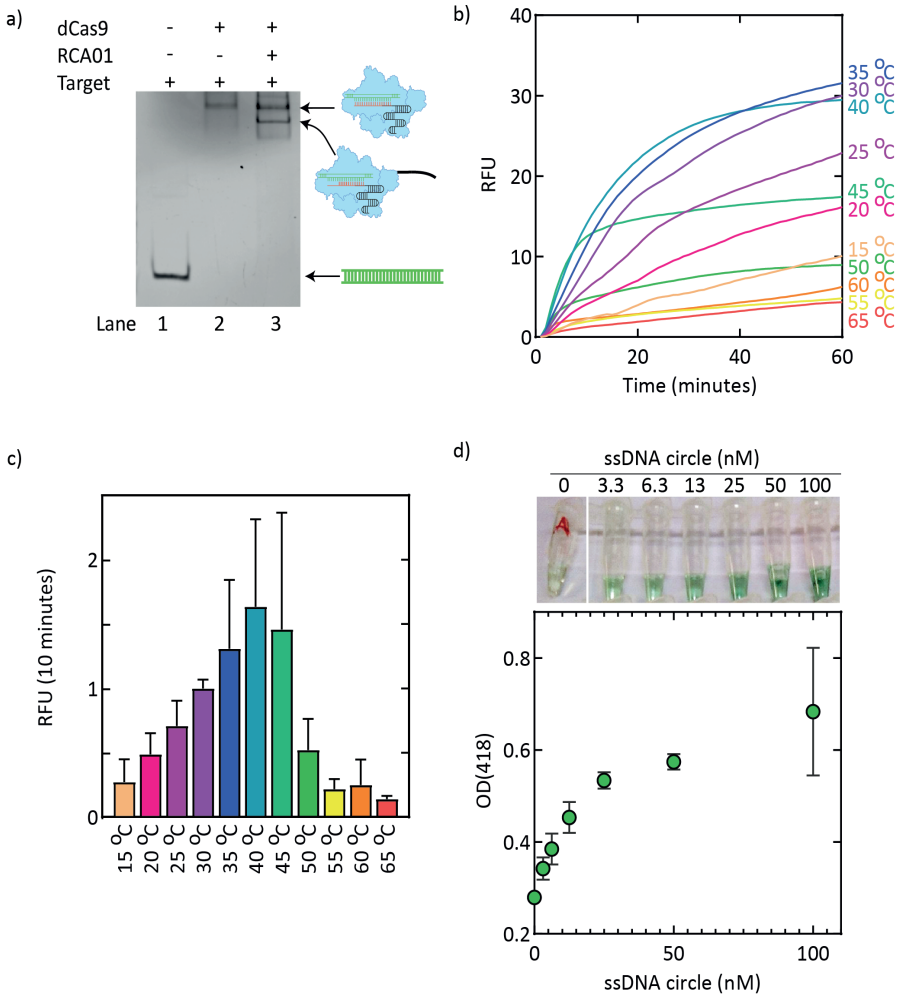

Figure 4.3: Detection of target DNA using CRISPR-dCas9 and rolling circle amplification (RCA). a) Representative shift assay with target DNA incubated either alone (lane 1), with sgRNA-dCas9 (lane 2), or with sgRNA-dCas9-RCA01 (lane 3). The lower-mobility band in lane 3, represents some dCas9 proteins without RCA01 (see also Supplementary Figure 5). b) Fluorescence versus time for RCA reactions at temperatures between 15°C and 60°C. c) Fluorescence after 10 minutes of the RCA reaction versus temperature. d) OD_{418} versus ssDNA circle concentration (nM) for RCA reactions at room temperature (23°C) after 24 hours. A representative picture of the resulting colour is shown above the graph.

In a next step, we employed the high sequence-specificity of the CRISPR-dCas9 system to further enhance the specificity of the targeting of pathogenic DNA. To subsequently couple the DNA detection by CRISPR-dCas9 to the colorimetric readout,

the dCas9 protein was covalently linked to a DNA oligonucleotide (named RCA01) which served as a primer for an RCA reaction (Figure 4.3a). To demonstrate that the dCas9-RCA01 complex efficiently bound to the RPA-amplified target DNA, we performed an electrophoretic mobility shift assay (EMSA). sgRNA was preincubated with the dCas9-RCA01 complex to form a sgRNA-dCas9-RCA01 complex, which was then incubated with the target DNA. The band shifts in the EMSA showed that the sgRNA-dCas9-RCA01 complex binds to the target DNA (lanes 2 and 3 in Figure 4.3a) with respect to the unbound target DNA (lane 1 in Figure 4.3a). An excess of dCas9-RCA01 over target DNA was used in the reaction to ensure that all target DNA was bound by the dCas9-RCA01 (Supplementary Figure 4.4).

In a second isothermal amplification step, RCA01 was hybridized to a circular RCA template, with the purpose to prime an RCA reaction. RCA was thus used for amplification to yield a long DNA molecule that contained many tandem repeats of G-quadruplexes, which in turn yielded a signal for a colorimetric readout. To make the circular ssDNA, we used a 109-mer linear oligonucleotide (named RCA02) with a sequence that encodes four tandem repeats of the G-quadruplex structure, plus a 20-mer linear oligonucleotide (named RCA03) that served as a bridging oligonucleotide that hybridizes to the two ends of RCA02 to facilitate ligation of these ends by T4 ligase to covalently close the circular template. This template design was selected as it facilitated efficient circularization by ligation (Supplementary Figure 4.6). The resulting ssDNA-circle sample was exonuclease digested to remove unligated templates and other single stranded oligonucleotides. The RCA01 oligonucleotide primed the RCA reaction and yielded a massive RCA product (with a linear length of 200nm to 5µm)[47,48] that contained a repetitive sequence complementary to that of the ssDNA circle. Figure 3 depicts the results of the RCA reaction. DNA production was monitored from the SYBR Green I fluorescence signal that was produced over 60 minutes. A wide range of temperatures was tested. RCA could successfully be conducted at all temperatures between 15C and 60°C, while it was optimal for temperatures of 25-40°C (Figure 4.3b, c).

The resultant RCA product encodes for G-quadruplexes which have peroxidase activity when they are in complex with hemin[49]. Upon the addition of hemin, $ABTS^{2-}$, and hydrogen peroxide to the final RCA product which contains an increasing amount of G-quadruplex DNA due to the RCA reaction, it will thus change in colour over time, as the hemin binds to the G-quadruplexes and facilitates the conversion of $ABTS^{2-}$ into the coloured $ABTS^{\bullet-}$ in the presence of the hydrogen peroxide. This change in colour is visible to the naked eye. Indeed, following extensive RCA for 24h at room temperature (23°C), reagents for the colour reaction (hemin, $ABTS^{2-}$, and hydrogen peroxide) were added, and the resulting colour change after 15 minutes was clear in the optical density OD_{418} as well as to the naked eye (Figure 4.3d). Notably, the RCA experiment shown in Figure 4.3d was conducted at room

temperature in the absence of any equipment, exemplifying the broad applicability of the assay.

While this CRISPR-dCas9-based DNA detection scheme may find a wide range of applications, we here show one example that is geared at PoC testing in resource-limited settings of parasitic DNA from VL. We selected kinetoplast minicircle DNA as the target, as a single parasite contains about 10,000 copies of this minicircle DNA (Figure 4.4a).

4.3.3 Favorable target sequences can be identified in the *Leishmania* genome

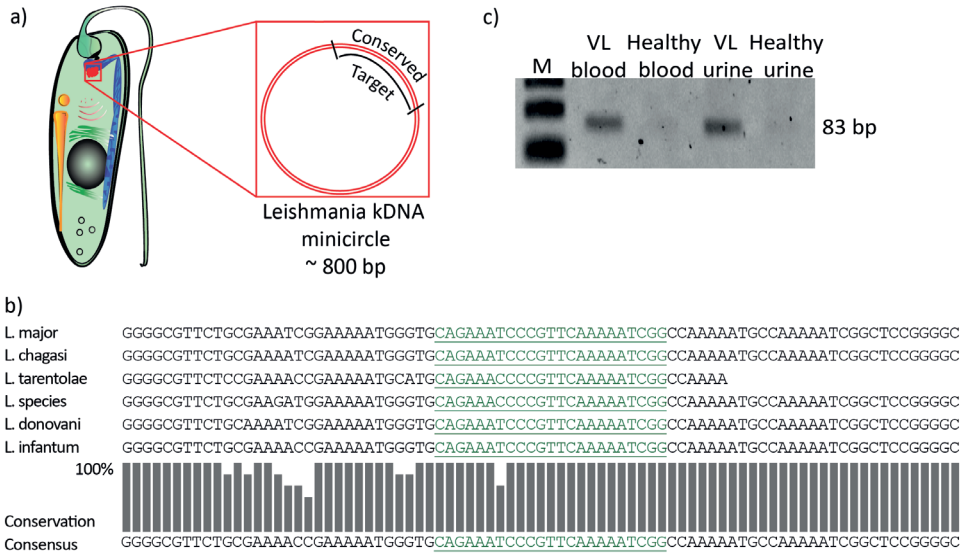

Figure 4.4: Target selection for diagnosis of Visceral Leishmaniases. a) Schematic of a *Leishmania* parasite. Inset: Kinetoplast minicircle DNA that was identified (~10,000 copies per cell) as a target. b) Target sequence identification: A multiple-alignment tool was used to identify a consensus region across the pan-leishmania genus that could serve as a potential target. Black letters denote the 115bp largely conserved sequence. Multiple iterations yielded putative targets within the kinetoplast minicircle DNA for recognition of L. major, L. chagasi, L. infantum, L. donovani, and L. tarentolae. The green letters denote a conserved 23-mer sequence that was selected as the final target, as it can serve as a CRISPR-dCas9 binding sequence, and as it has no homology with the human genome and non-VL pathogenic genomes. c) PCR showing the presence of the VL target in patient blood and urine, Lane 1 (M): 50 bp DNA ladder showing 50, 100, 150 bp oligomers. Lane 2: PCR amplified target (83 bp) from VL patient's blood sample. Lane 3: healthy blood (DNA extracted using kit from 500 µl of blood). Lane 4: PCR amplified target (83 bp) from VL patient's urine sample, lane 5: healthy urine (circulating cell free DNA extracted using kit from 13 ml of urine).

To identify a specific consensus region across all the leishmania species within the ~800 bp kinetoplast minicircle, a multiple sequence alignment tool called T-coffee was used[50]. To avoid misdiagnoses and false positive results, the identified consensus sequences were further analysed for homologies against other pathogen's genomes, including Trypanosoma and Plasmodium species which co-exist in VL-endemic regions, and against the human genome using BLAST[50]. No significant homologies were found.

A potential target sequence of 115 bp was identified that contained the dCas9 protospacer adjacent motif (PAM) recognition site (NGG for Streptococcus pyogenes Cas9) (Figure 4.4b and Supplementary Figure 4.8). To further verify if the target gene was present in patient's samples such as blood and urine, a PCR was performed on DNA extracted from a VL patient's blood and urine sample, as well as from a blood and urine sample from healthy humans as controls, using a set of primers that yielded an 83 bp DNA product that is present within the 115 bp consensus target sequence (Figure 4.4c). The results confirmed the presence of the target DNA in the VL patient blood and urine samples.

Finally, we performed the process from the RPA reaction to the colorimetric readout using a VL target sequence for optimization (Figure 4.5). Using an input of 10^{11} VL target molecules, amplified biotinylated target DNA (post-RPA) was immobilized on streptavidin-coated beads, followed by subsequent dCas9 detection, and isothermal amplification by RCA (using the circular template RCA04) at room temperature (23°C) for 24 hours to produce a colorimetric readout. The results demonstrate a clear visual readout in the sample containing the target DNA compared to the negative sample (without target DNA) (Figure 4.5).

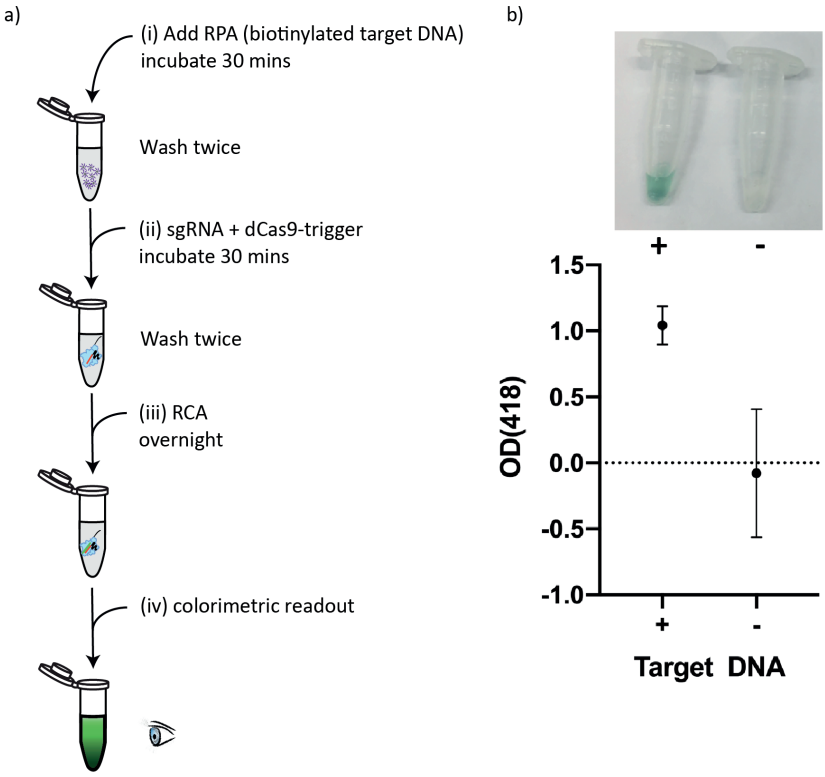

Figure 4.5: Workflow of the DNA detection scheme.

a) Schematic representation of the detection of amplified biotinylated-target DNA with a colorimetric readout, where amplified biotinylated-target DNA (post-RPA) is immobilized on streptavidin-coated beads, followed by subsequent dCas9 detection, and isothermal amplification by RCA to produce a colorimetric readout. b) OD_{418} detection with and without target DNA (N=3, errors are SD) after processing at room temperature (23℃) for 24 hours. The values were normalized to a negative control that contained streptavidin-coated beads in a 1X reaction buffer. A representative picture of the resulting colour is shown Figure 4.5b, right.

4.3 Discussion and conclusion

We have developed a novel DNA detection scheme that is broadly applicable for biosensing applications such as diagnostics. Target DNA can be isolated from biological samples sensitively, specifically, rapidly, and across a broad temperature range. Target DNA is amplified, bound by CRISPR-dCas9, amplified further, and visualized with a colorimetric readout that is visible with a naked eye. The ability of the dCas9-RCA01 complex to bind to the RPA-amplified target DNA ensures the specificity and robustness of the direct DNA-detection scheme. The visible readout to the naked eye and functionality at room temperature throughout all extraction and detection steps confer specific advantages for facile application.

We thus demonstrated an innovative DNA-detection scheme that is suitable for disease diagnosis in resource-limiting settings. Since we aim to apply our DNA detection scheme as an instrument-free diagnostic test, we first utilized a pH-based chitosan-mediated DNA-extraction method. Our results demonstrated the high sensitivity of this DNA extraction method, and suggest that it can be applied to detect even a single parasite in a pin-prick of blood (~10μL) or fewer than 10 copies of circulating cell-free DNA in a urine sample from a patient. Notably, since 'room temperature' can differ substantially in the global South where many NTDs remain endemic[51], it is advantageous that the isothermal reactions used in this detection scheme perform efficiently across a wide range of temperatures up to 40°C. Notably, our isothermal DNA extraction method does not employ any temperatures beyond room temperature, which contrasts other DNA detection platforms for resource-limiting settings, such as HUDSON which relies on heating the samples to extract DNA before detection by CRISPR-Cas[36].

Due to the ease of programmability of the CRISPR-dCas9 system, this DNA detection scheme will be broadly applicable as it may be programmed to detect any pathogenic DNA, genetic variants (SNPs, insertions, deletions), and antimicrobial resistant strains, as well as be used in other biosensing applications such as forensics and genotyping, for example to facilitate self-screening for diseases. Upon further development, our novel direct DNA detection scheme can also be multiplexed for diseases that show overlapping symptoms. For example, VL is often misdiagnosed as it presents overlapping symptoms with other febrile illnesses such as malaria[24]. Additionally, multiplexing capabilities to co-detect other conditions that will change the treatment procedure, such as HIV and pregnancy, is a possibility with our novel direct DNA-detection scheme.

The diagnostic scheme presented in this study is compatible with lyophilization, allowing this direct DNA-detection scheme to be packaged into a completely

closed, fully or semi-automated microfluidic device with sample-in answer-out capabilities for testing blood or urine samples as a field- or home-deployable diagnostic test (Supplementary Figure 4.8). In addition to providing a valuable tool in epidemics, such a test that could facilitate diagnosis at homes would be of great use for diagnosis of the persistent NTDs, which affect more than 1 billion people worldwide and constitute a significant global health problem[15]. For most NTDs, diagnostic tests are ineffective due to a lack of resources and/or expert personnel (see Chapter 2). This need for field-deployable PoC diagnostics can be addressed by our sensitive, specific, user-friendly, rapid, robust, and equipment-free direct DNA-detection scheme. This confirmatory diagnostic test could potentially replace cumbersome culturing and microscopy-based diagnostic procedures by providing accurate real-time results at the PoC. Since our DNA-detection scheme can function independently of the patients' immune response, it can be applied to all ethnic populations and present a test-of-cure and test-of-relapse of infections. Owing to its enhanced specificity, the combination of RPA and CRISPR/dCas9 detection used in this DNA detection scheme can be expected to prevent false positives and hence outcompete current antibody-based rapid diagnostic tests.

Summing up, the novel detection scheme presented in this study is advantageous over other NA-detecting methods, as it does not require electricity, advanced equipment, or expert personnel to operate, and it can be designed to sensitively detect a broad range of DNA targets. We anticipate that this simple, specific, and sensitive diagnostic scheme can be readily applied to address the diagnostic PoC needs of various infectious diseases and thus help alleviate the global healthcare burden.

4.4 Materials and Methods

Target selection and optimization

A multiple-alignment tool (T-coffee software, tcoffee.crg.cat) was used to identify a consensus region across the pan-*Leishmania (*L) genus that could serve as a potential target. Multiple iterations yielded putative targets within kinetoplast minicircle DNA for recognition of *L*. *major*, *L*. *chagasi*, *L*. *infantum*, *L*. *donovani*. *L*. *tarentolae* and *L*. *amazonensis*. The identified targets were further checked for homology with human or non-*L* disease-causing pathogen's sequences using BLAST. A sequence of 115 bp was identified that contained a 23-mer CRISPR-dCas9 target that had no homology with other genomes (see supplementary figure 4.7). The synthetic gene was cloned into pUC 57 plasmid (GenScript (Leiden, Netherlands)) and transformed in *E. coli* top10 cells. DNA was extracted using a Qiagen plasmid midi kit and the synthetic target gene construct was obtained using standard Phusion DNA polymerase PCR employing primers pairs, synthetic *Leishmania* target forward (FWD) primer and synthetic *Leishmania* target reverse (REV) primer, employing the following protocol: 98°C for 3 minutes followed by 30 cycles of [98°C for 10 seconds, then 58°C for 20 seconds, and 72°C for 15 seconds], with a final hold of 72°C for 8 minutes. PCR product (target DNA) was checked on 3% agarose gel and further cleaned using an NEB monarch kit. For target detection within VL patient's blood and urine samples, primer pairs (VL target FWD PCR primer and VL target REV PCR primer) were used for standard Phusion DNA polymerase PCR (same protocol as above). The template for PCR was obtained using the genomic DNA extraction kit (Qiagen, Europe) utilizing 500 µl of VL patient's blood, and circulating cell free DNA extraction kit (Qiagen, Europe) utilizing 13 ml of VL patient's urine.

Chitosan-based DNA extraction

10 cm long Fusion-5 filter paper strips were plasma-cleaned (2-3 minutes) prior to overnight incubation in chitosan solution (0.05% w/v in 0.1% acetic acid, pH 6.0) at room temperature. Thereafter, chitosan-functionalized paper was washed three times with deionized water, dried at 60°C for 1 hour and stored at room temperature. 10 mM MES buffer (pH 5.0) supplemented with target DNA (at different concentrations) was used to mimic patient's sample. To entrap the DNA, 10 µl of MES buffer with target DNA was added to 6 ± 0.5 mm sized paper-discs of chitosan-functionalized paper and incubated for 5 minutes. To elute the target DNA, 20 µl of 50 mM Tris(hydroxymethyl)aminomethane (Tris) (pH 7.9) or 20 µl of the rehydration buffer from the RPA kit was added directly unto the DNA entrapped chitosan-functionalized paper. The eluate was used as a template for the subsequent isothermal DNA amplification.

Isothermal DNA amplification

Recombinase polymerase amplification was employed to obtain multiple copies of target DNA using the Twist Dx basic kit as per the manufacturer's instructions. The primer sequences, VL target FWD RPA and VL target REV RPA, were designed outside the CRISPR-dCas9 target region. Note that for Figure 5, a biotinylated VL target FWD RPA primer and VL target REV RPA was used.

sgRNA production

To make the single guide gRNA (sgRNA), we first PCR amplified a dsDNA template, which contained the consensus sequence from a DNA plasmid (pgRNA-bacteria plasmid from Addgen), using a sgRNA FWD primer that contained a T7 promoter, and sgRNA REV primer. The following thermal cycling conditions were used to generate the PCR template: 98°C for 3 minutes; 98°C for 10 seconds; 65°C for 20 seconds; 72°C for 15 seconds; go to 98°C for 10 seconds; 65°C for 20 seconds; 72°C for 15 seconds for 29 cycles and 72°C for 8 minutes. The PCR template was verified using gel electrophoresis (1,5% agarose, 1X TBE buffer, 120V for 90 minutes) and subsequently purified using the WizardSV Gel and PCR Clean-Up System (Promega) according to the manufacturer's instructions. sgRNA was then transcribed from the PCR template using the RiboMaxTM Large Scale RNA Production Systems kit (Promega) according to the manufacturer's instructions. Following transcription, RNA products were purified using the RNeasy MinElute Cleanup Kit (Qiagen) according to the manufacturer's instructions. RNA quality was verified using gel electrophoresis (Mini-Protean TBE-Urea Precast Gels (Bio-Rad), 200V for 30 minutes). Gels were visualized under UV light in a Biorad ChemiDOCT MP imaging system.

sgRNA-dCas9-oligonucleotide complex assembly

To covalently attach an oligonucleotide to the dCas9 protein, an oligonucleotide sequence (RCA01) that was modified with a 5' O_6-benzylguanine (BG) group (Biomers) was incubated with the dCas9-Snap protein at 37°C for 60 minutes. The dCas9-RCA01 complex was then purified using the AKTA pure chromatography system. We then assembled sgRNA, dCas9-RCA01, and DNA in a 1× NEBuffer 3.1 Reaction Buffer (New England Biolabs, 100 mM NaCl, 50mM Tris-HCl, 10 mM $MgCl_2$, 100ug/mL BSA, pH 7.9 @ 25 °C) in a molar ratio of 100:10:1 (sgRNA/dCas9-RCA01/DNA). An excess ratio of dCas9-RCA01 was used to ensure maximum binding of the DNA to the protein. sgRNA was prepared by heating up to 95°C for 10 minutes and slowly cooling down (1°C every 4 minutes until a final temperature of 4°C). sgRNA was then incubated with dCas9-RCA01 at 25°C for 30 minutes. sgRNA- dCas9-RCA01 complexes were then incubated with DNA at 37°C for 30 minutes. The binding

affinity of the sgRNA-dCas9-RCA01 complexes to the DNA was verified using an Electrophoretic Mobility Shift Assay (EMSA) (10% 1X TBE-Precast Gels (Invitrogen), 90V for 90 minutes). Gels were stained with Ethidium Bromide and visualized under UV light in a Biorad ChemiDOCT MP imaging system.

Production of the circular RCA template and isothermal RCA reaction

The RCA template was produced using a template oligonucleotide RCA02. RCA02 was 5'-phosphorylated by T4 PolyNucleotide Kinase (PNK) for a final concentration of 1 µM and 0.1 units/µL, respectively. The 5'-phosphorylation reaction was performed for 60 minutes using 1x PNK buffer supplied by the manufacturer and 500 µM ATP. Following the 5'-phosphorylation, a primer oligonucleotide RCA03 was added for a final concentration of 3 µM, before all secondary structures in the DNA were disrupted by incubation at 95 °C for 10 minutes. The solution was allowed to cool to room temperature, before fresh ATP and T4 Ligase was added to the solution obtaining a concentration of 100 µM ATP and 0.4 units/µL T4 ligase, and the reaction proceeded for 16 hours at room temperature. Note that for figure 5, a template oligonucleotide RCA04 was used, with the primer oligonucleotide RCA03. The resulting circular template with primer was either used directly for RCA or stored at -20 °C. RCA was performed using a final concentration of 0.1 units/µL phi29 polymerase and 80 µM of nucleotides. The RCA reaction was performed at 30 °C for 30 minutes, unless indicated otherwise in the text. RCA products were visualised on 1% (w/v) agarose gels.

Colorimetric readout

To visualise the products of the RCA reaction with the naked eye, 0.6 µl of 100 µM hemin, 2 µL of 50 mM 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS) and 1.8 µL of 40 mM H_2O_2 was added to the RCA reaction at room temperature and left to incubate for 5 minutes. Thereafter, the colour change was recorded with a digital camera and the absorbance/OD was measured at 418nm.

4.5 Supplementary Information

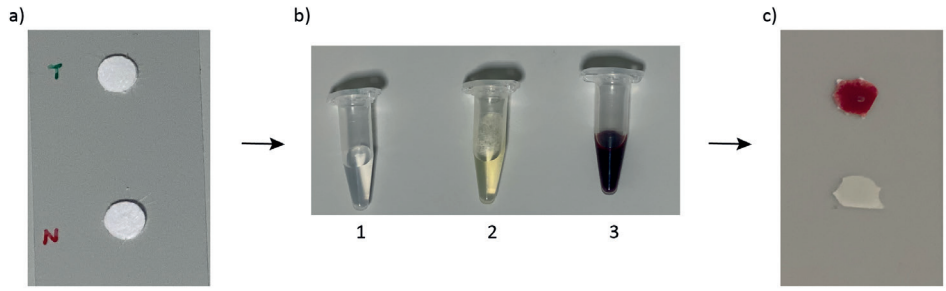

Figure S4.1: pH-based chitosan-mediated scheme to extract target DNA. a) chitosan-functionlized fusion-5 paper discs (T=target; N=negative control). b) Target DNA is spiked into acidic buffer (1), urine (2), or healthy blood sample (3) and each fluid (pH 5.0.) is administered onto the chitosan-functionlized fusion-5 paper discs as seen in panel a. Buffer, blood, or urine without target DNA are used as negative controls. c) The fusion-5 paper discs are placed inside the tubes (panel b) and washed with alkaline buffer (pH 8.0), to yield paper discs as seen in panel c. The DNA-containing elutes were further processed.

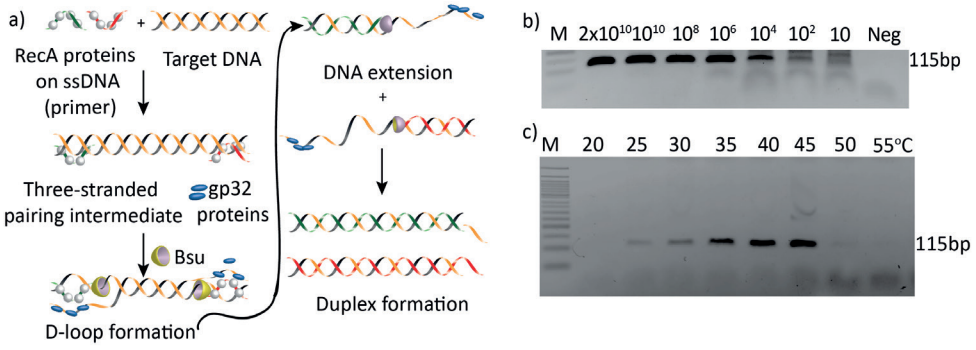

Figure S4.2: Isothermal target DNA amplification. a) Schematic of the working principle behind Recombinase Polymerase Amplification (RPA). Adapted from52. b) To determine the limit of detection for the RPA reaction, a titration of input target DNA from 2x1010 VL target molecules to 10 VL target molecules was amplified at 39°C for 30 mins. RPA can detect as few as 10 VL target molecules. c) To determine the operating temperature of the RPA reaction, RPA was then performed from 20°C to 55°C. RPA can amplify the target DNA across a broad temperature range from 25°C to 45°C.

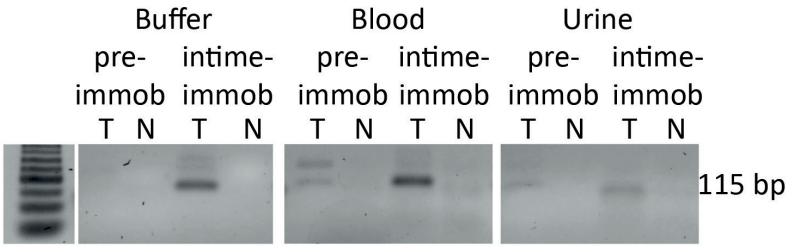

Figure S4.3: Streptavidin-biotin immobilization of target DNA to a streptavidin-coated surface either before ('pre-immob') or during ('intime immob') the RPA reaction. To determine if biotinylated-RPA primers should be immobilized to a streptavidin-coated surface before or during an RPA reaction, spiked-target DNA was extracted from buffer, blood or urine samples, and subsequently amplified by RPA with biotinylated-primers that were either immobilized to a streptavidin-coated surface before or during an RPA reaction. T denotes samples that contain spiked-target DNA. N denotes negative controls that do not contain spiked-target DNA. The results demonstrate that biotinylated-RPA primers should be immobilized to a streptavidin-coated surface during an RPA reaction for buffer samples. For both blood and urine samples, biotinylated-RPA primers could be immobilized to a streptavidin-coated surface before or during an RPA reaction, but more RPA amplicons were produced when biotinylated-RPA primers were immobilized during an RPA reaction. These results have implications for the final diagnostic device, and strongly suggest that biotinylated-RPA primers should be immobilized to a streptavidin-coated surface during an RPA reaction ("intime").

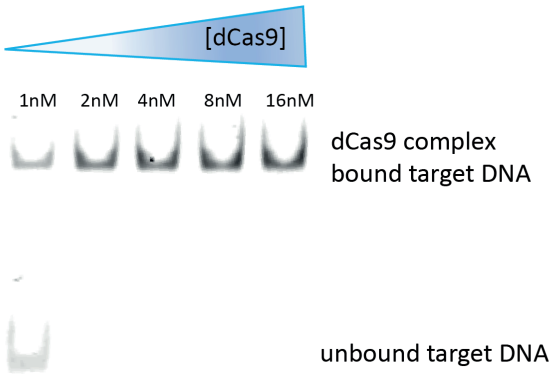

Figure S4.4: dCas9 binding to dsDNA target. EMSA using 1nM dsDNA target and increasing dCas9 concentrations (1nM-16nM). The upper band represents dCas9 bound to dsDNA while the lower band represent unbound dsDNA. An excess of dCas9 over target (minimum 2 times excess) is required to bind all the target DNA.

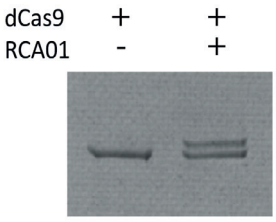

Figure S4.5: Protein gel analysis. dCas9 in the presence and absence of RCA01, lanes 1 and 2 respectively. The double bands in the presence of RCA01 shows that the covalent attachment of the oligonucleotide RCA01 is partially efficient. The upper band represents the proportion of dCas9 that is covalently attached to RCA01.

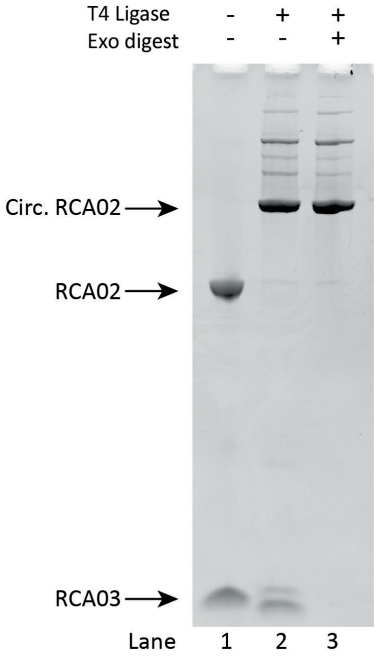

Figure S4.6: Circular ligation efficiency. To determine the ligation efficiency of the circularization technique used in this study, RCA02 and RCA03 were analysed in a denaturing gel. Lane 1 shows the input oligonucleotides RCA02 and RCA03 prior to any treatment. Lane 2 shows RCA02 and RCA03 after T4 ligation. Lane 3 shows RCA01 and RCA02 after T4 ligation and exonuclease treatment ("exo digest"). After the T4 ligation treatment, the RCA02 band disappeared while other low-mobility bands appeared (lane 2). After exonuclease treatment, the RCA03 bands disappeared, whereas the low-mobility bands remained (lane 3). These exonuclease-resistant low-mobility bands most likely represent larger circles of oligomers of RCA02. Most importantly, single circularized RCA02 was the major product ("circ. RCA02").

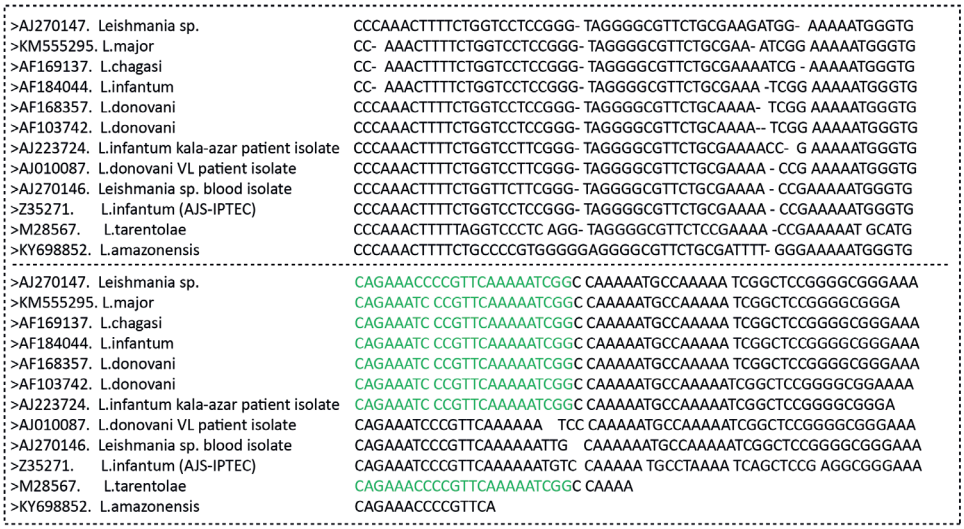

Figure S4.7: Target selection for the diagnosis of Leishmaniases. Overview (screenshot) of a multiple sequence alignment analysis that shows the unique identifying sequence code per leishmania species isolate (far left), and the resulting sequence (right) which is plotted across the top and bottom columns. Multiple alignment tool (T-coffee software, tcoffee.crg.cat) was used to identify a consensus region across the pan-leishmania genus that could serve as a potential target for detection by CRISPR-dCas9. Multiple iterations yielded putative targets within the kinetoplast minicircle DNA for recognition of *L. major, L. chagasi, L. infantum, L. donovani. and L. tarentolae*. Using BLAST, the identified targets were further checked for nonhomology with human or other pathogen's sequence. A conserved sequence of 115bp (full sequences denoted) was identified, that contains a 23-mer CRISPR-dCas9 target site (green, underlined) that has no homology with other genomes.

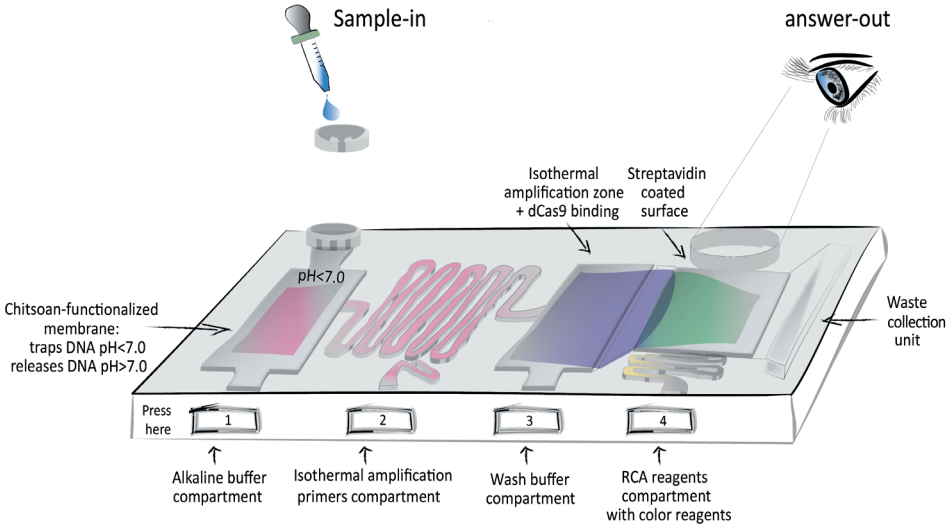

Figure S4.8: Possible microfluidic packaging of the direct DNA sensor as a rapid diagnostic test. A simple DNA sensor with sample-in, answer-out capabilities could be packaged into a microfluidic device. Such an easy-to-perform test would come with an accurate binary result in the form of a colour in the control and test zones that is visible to the naked eye. While a colour in the control zone would ensure the functionality of the test, an appearance of the test colour at the probing site would confirm a positive result, i.e., the presence of pathogen's DNA in the biological sample that was applied.

Supplementary table

Synthetic *Leishmania* target FWD primer	5' CCCAAACTTTTCTGGTCCTCCG 3'
Synthetic *Leishmania* target REV primer	5' TTTCCCGCCCCGGAGC 3'
VL target FWD PCR primer	5' GGGGCGTTCTGCGAAGA 3'
VL target REV PCR primer	5' GCCCCGGAGCCGAT 3'
VL target FWD RPA primer	5' CCCAAACTTTTCTGGTCCTCCGGGTAGGGGC
VL target REV RPA primer	5' TTTCCCGCCCCGGAGCCGATTTTTGGCATT
Biotinylated VL target FWD RPA primer	5' Biotin/TTTTTTGAATTCCCCAAACTTTTCTGGTCCTCCGGGTAGGGGC 3'
sgRNA FWD primer	5' TAATACGACTCACTATAGGCAGAAACCCCGTTCAAAAATGTTTTAGAGCTAGAAATAGC AAGTTAAAATAA GG 3'
sgRNA REV primer	5' AAAAAAAGCACCGACTCGGTGCCAC 3'
RCA01	5' TTTTTTTTTTTACATGCTCGAGATCAGTTTTTTATGCGCCTGTTGCC 3'
RCA02	5' CTACTACCTCACCTCACCCAACCCGCCCTACCCAAAACCCAACCCGCCCTACCCAAAAC CCAACCCGCCCTACCCAAAAGGCAACAGGCGCATAAAACAACTATACAAC 3'
RCA03	5' GAGGTAGTAGGTTGTATAGT 3'
RCA04	5' CTACTACCTCACCTCACCCAACCCGCCCTACCCAAAACCCAACCCGCCCTACCCAAAAC CCAACCCGCCCTACCCAAAAGGCAACAGGCGCATAAAACACCT CAGCACTATACAAC 3'

References

1. Du, F. *et al.* transcription PCR and strand-displacement based signal. 1544–1548 (2013). doi:10.1039/c2an36688f.
2. Zhao, Y., Zhou, L. & Tang, Z. Cleavage-based signal amplification of RNA. 1–8 (2013). doi:10.1038/ncomms2492.
3. Foudeh, A. M., Fatanat Didar, T., Veres, T. & Tabrizian, M. Microfluidic designs and techniques using lab-on-a-chip devices for pathogen detection for point-of-care diagnostics. *Lab Chip* **12**, 3249–3266 (2012).
4. Fluit, A. C., Visser, M. R. & Schmitz, F. J. Molecular detection of antimicrobial resistance. *Clin. Microbiol. Rev.* **14**, 836–871 (2001).
5. Seyerle, A. A. & Avery, C. L. Genetic epidemiology: the potential benefits and challenges of using genetic information to improve human health. *N. C. Med. J.* **74**, 505–508 (2013).
6. Butler, J. M. The future of forensic DNA analysis. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **370**, 20140252 (2015).
7. Gomez, A., Miller, N. S. & Smolina, I. Visual Detection of Bacterial Pathogens via PNA-Based Padlock Probe Assembly and Isothermal Ampli fi cation of DNAzymes. (2014).
8. Vidic, J. *et al.* Point-of-Need DNA Testing for Detection of Foodborne Pathogenic Bacteria. *Sensors (Basel).* **19**, 1100 (2019).
9. Wee, E. J. H., Lau, H. Y., Botella, J. R. & Trau, M. Re-purposing bridging flocculation for on-site, rapid, qualitative DNA detection in resource-poor settings. *Chem. Commun.* **51**, 5828–5831 (2015).
10. Zhao, W., Ali, M. M., Aguirre, S. D., Brook, M. A. & Li, Y. Paper-Based Bioassays Using Gold Nanoparticle Colorimetric Probes. *Anal. Chem.* **80**, 8431–8437 (2008).
11. Wang, Q., Zhang, B., Xu, X., Long, F. & Wang, J. CRISPR-typing PCR (ctPCR), a new Cas9-based DNA detection method. *Sci. Rep.* **8**, 1–13 (2018).
12. Zhou, L., Peng, R., Zhang, R. & Li, J. The applications of CRISPR/Cas system in molecular detection. *J. Cell. Mol. Med.* **22**, 5807–5815 (2018).
13. Plesivkova, D., Richards, R. & Harbison, S. A review of the potential of the MinION™ single-molecule sequencing system for forensic applications. *WIREs Forensic Sci.* **1**, e1323 (2019).
14. Yang, W. *et al.* Detection of CRISPR-dCas9 on DNA with Solid-State Nanopores. *Nano Lett.* **18**, 6469–6474 (2018).
15. Hotez, P. J., Pecoul, B., Rijal, S., Boehme, C. & Aksoy, S. Eliminating the Neglected Tropical Diseases : Translational Science and New Technologies. 1–14 (2016). doi:10.1371/journal.pntd.0003895.
16. Mitra, A. K. & Mawson, A. R. Neglected tropical diseases: Epidemiology and global burden. *Trop. Med. Infect. Dis.* **2**, (2017).
17. Singh, O. P. & Sundar, S. Review Article Developments in Diagnosis of Visceral Leishmaniasis in the Elimination Era. **2015**, (2015).
18. Park, S., Zhang, Y., Lin, S., Wang, T.-H. & Yang, S. Advances in microfluidic PCR for point-of-care infectious disease diagnostics. *Biotechnol. Adv.* **29**, 830–839 (2011).
19. Roskos, K. *et al.* Simple System for Isothermal DNA Amplification Coupled to Lateral Flow Detection. **8**, (2013).
20. Li, Y., Li, S., Wang, J. & Liu, G. CRISPR/Cas Systems towards Next-Generation Biosensing. *Trends Biotechnol.* **37**, 730–743 (2019).

21. Abeijon, C. *et al.* Development of a multiplexed assay for the detection of *Leishmania donovani/Leishmania infantum* protein biomarkers in the urine of patients with visceral leishmaniasis. *J. Clin. Microbiol.* (2019). doi:10.1128/JCM.02076-18.
22. Biosensing, N., Li, Y., Li, S., Wang, J. & Liu, G. CRISPR / Cas Systems towards. 1–14 (2019).
23. Kozel, T. R. & Burnham-Marusich, A. R. Point-of-Care Testing for Infectious Diseases: Past, Present, and Future. *J. Clin. Microbiol.* **55**, 2313 LP – 2320 (2017).
24. Burza, S., Croft, S. L. & Boelaert, M. Seminar Leishmaniasis. **392**, 951–970 (2018).
25. Abeijon, C. *et al.* Development of a Multiplexed Assay for Detection of Leishmania donovani and Leishmania infantum Protein Biomarkers in Urine Samples of Patients with Visceral Leishmaniasis. *J. Clin. Microbiol.* **57**, e02076-18 (2019).
26. Schallig, B. Y. M. N. and H. D. F. H. Evaluation of Urine as a Diagnostic Specimen for Visceral Leishmaniasis in Sudan. *Journal of Tropical Diseases* **1**, 1–3 (2013).
27. Bezerra, G. S. N., Barbosa, W. L., Silva, E. D. da, Leal, N. C. & Medeiros, Z. M. de. Urine as a promising sample for Leishmania DNA extraction in the diagnosis of visceral leishmaniasis – a review. *Brazilian J. Infect. Dis.* **23**, 111–120 (2019).
28. Toley, B. J. *et al.* Isothermal strand displacement amplification (iSDA): A rapid and sensitive method of nucleic acid amplification for point-of-care diagnosis. *Analyst* **140**, 7540–7549 (2015).
29. Song, J. *et al.* Two-Stage Isothermal Enzymatic Amplification for Concurrent Multiplex Molecular Detection. *Clin. Chem.* **63**, 714–722 (2017).
30. Zhou, W. *et al.* A CRISPR–Cas9-triggered strand displacement amplification method for ultrasensitive DNA detection. *Nat. Commun.* **9**, 5012 (2018).
31. Pardee, K. *et al.* Rapid, Low-Cost Detection of Zika Virus Using Programmable Biomolecular Components. *Cell* **165**, 1255–1266 (2016).
32. Batista, A. C. & Pacheco, L. G. C. Detecting pathogens with Zinc-Finger, TALE and CRISPR-based programmable nucleic acid binding proteins. *J. Microbiol. Methods* **152**, 98–104 (2018).
33. Gootenberg, J. S. *et al.* Multiplexed and portable nucleic acid detection platform with Cas13, Cas12a and Csm6. *Science (80-.).* **360**, 439–444 (2018).
34. Choi, J. R. *et al.* An integrated lateral flow assay for effective DNA amplification and detection at the point of care. *Analyst* **141**, 2930–2939 (2016).
35. Chiu, C. In Translation Cutting-Edge Infectious Disease Diagnostics with CRISPR. 2015–2017 (2018).
36. Myhrvold, C. *et al.* Field-deployable viral diagnostics using CRISPR-Cas13. *Science (80-.).* **360**, 444–448 (2018).
37. Murakami, T., Sumaoka, J. & Komiyama, M. Sensitive isothermal detection of nucleic-acid sequence by primer generation-rolling circle amplification. *Nucleic Acids Res.* **37**, (2009).
38. Bezuneh, A. *et al.* Comparison of point-of-care tests for the rapid diagnosis of visceral leishmaniasis in East African patients. *Am. J. Trop. Med. Hyg.* **91**, 1109–1115 (2014).
39. Schallig, H. D. F. H. *et al.* Evaluation of point of care tests for the diagnosis of cutaneous leishmaniasis in Suriname. *BMC Infect. Dis.* **19**, 25 (2019).
40. Buy isothermal DNA/RNA amplification products | TwistDx. Available at: https://www.twistdx.co.uk/en/products. (Accessed: 29th April 2020)
41. Cao, W., Easley, C. J., Ferrance, J. P. & Landers, J. P. Chitosan as a Polymer for pH-Induced

DNA Capture in a Totally Aqueous System. *Anal. Chem.* **78**, 7222–7228 (2006).
42. Ereku, L. T. *et al.* RPA using a multiplexed cartridge for low cost point of care diagnostics in the field. *Anal. Biochem.* **547**, 84–88 (2018).
43. Okwor, I. & Uzonna, J. Social and Economic Burden of Human Leishmaniasis. *Am. J. Trop. Med. Hyg.* **94**, 489–493 (2016).
44. Aronson, N. *et al.* Diagnosis and Treatment of Leishmaniasis: Clinical Practice Guidelines by the Infectious Diseases Society of America (IDSA) and the American Society of Tropical Medicine and Hygiene (ASTMH). *Clin. Infect. Dis.* **63**, 1539–1557 (2016).
45. Chappuis, F. *et al.* Diagnostic Accuracy of Two rK39 Antigen-Based Dipsticks and the Formol Gel Test for Rapid Diagnosis of Visceral Leishmaniasis in Northeastern Uganda Diagnostic Accuracy of Two rK39 Antigen-Based Dipsticks and the Formol Gel Test for Rapid Diagnosis of Vis. *J. Clin. Microbiol.* **43**, 5973–5977 (2005).
46. Gan, W. *et al.* Chitosan-Modified Filter Paper for Nucleic Acid Extraction and "in Situ PCR" on a Thermoplastic Microchip. *Anal. Chem.* **89**, 3568–3575 (2017).
47. Zhu, G. *et al.* Noncanonical Self-Assembly of Multifunctional DNA Nanoflowers for Biomedical Applications. *J. Am. Chem. Soc.* **135**, 16438–16445 (2013).
48. Franch, O. *et al.* DNA flowerstructure co-localizes with human pathogens in infected macrophages. *Nucleic Acids Res.* **48**, 6081–6091 (2020).
49. Cheng, X., Liu, X., Bing, T., Cao, Z. & Shangguan, D. General Peroxidase Activity of G-Quadruplex−Hemin Complexes and Its Application in Ligand Screening. *Biochemistry* **48**, 7817–7823 (2009).
50. Daugelaite, J., O' Driscoll, A. & Sleator, R. D. An Overview of Multiple Sequence Alignments and Cloud Computing in Bioinformatics. *ISRN Biomath.* **2013**, 615630 (2013).
51. Lindoso, J. A. L., Cunha, M. A., Queiroz, I. T. & Moreira, C. H. V. Leishmaniasis-HIV coinfection: current challenges. *HIV. AIDS. (Auckl).* **8**, 147–156 (2016).
52. Piepenburg, O., Williams, C. H., Stemple, D. L. & Armes, N. A. DNA Detection Using Recombination Proteins. *PLOS Biol.* **4**, e204 (2006).

5

Small RNA molecules inhibit the catalytic activity of Cas9 in vitro

The Cas9 endonuclease from *Streptococcus pyogenes* (SpCas9) has been widely applied in genome editing. Despite the ease of application, the delivery of Cas9 to cells for therapeutics remains problematic. Most delivery methods include a plasmid-based approach where Cas9 and gRNA are encoded in the same vector, delivery of mRNA for Cas9 translation together with a separate gRNA, and the introduction of an assembled ribonucleoprotein complex. Of the three strategies, the first two are easier to deliver, however they suffer from the lack of control over Cas9 activity and expression, and are more susceptible to cellular factors that can decrease the activity of Cas9. Here we report that small RNA and DNA molecules efficiently inhibit the catalytic activity of Cas9 in vitro when hybridized with the guide RNA. We use single-molecule fluorescence to determine that Cas9 efficiently loads the duplex but is then stuck in an inactive conformation which prevents it from interacting with the target even after the inhibitor molecule is removed.

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5.1 Introduction

Clustered regularly interspaced short palindromic repeat (CRISPR) systems and their associated (Cas) proteins are adaptive prokaryotic immune systems that provide bacteria and archaea with adaptive immunity against invading viruses and plasmids[1-5]. The type II-A CRISPR/Cas9 system from *Streptococcus pyogenes* is a well characterized RNA-guided DNA endonuclease that has been extensively repurposed for genome engineering due to its simplicity and programmability[6-9]. However, efficient delivery of Cas9 to cells and tissue remains problematic. Most delivery methods rely on one of the three following approaches: delivery of the DNA encoding Cas9 and guide RNA (gRNA) using a viral vector, delivery of gRNA and mRNA for translating Cas9 and assembling the ribonucleoprotein (RNP) complex in vivo, and electroporating the assembled RNPs into cells[10-15]. While the third strategy offers the greatest control of the assembly of functional Cas9:RNA complexes and their concentration, it is limited to ex vivo applications. The other two main strategies can be applied in situ, however, they do not offer precise control over the expression of Cas9 and the assembly of functional RNPs. Furthermore, cellular components might interfere with either the RNA transcription, the translation of the protein, or RNA loading into Cas9. In particular, Cas9 has been demonstrated to be an efficient strategy for the treatment of HIV when applied in combination with RNA interference[16]. In such cases, if Cas9 is to be applied with a different RNA-guided protein targeting a similar gene, we postulate that the RNA sequences may undergo transient interactions via Watson-Crick base-pairing and may in turn affect the activity of Cas9 if the RNP is expressed and assembled in vivo. Here we demonstrate that small RNA molecules can completely abolish the target cleavage of Cas9 in vitro, and use single-molecule fluorescence microscopy in order to determine the mechanism of inhibition.

5.2 Results

In order to determine if small RNAs have an effect on Cas9 function, we designed inhibitor RNA molecules complementary to either the PAM proximal or PAM distal regions of the single guide RNA (sgRNA) (Figure 5.1a). The sgRNA was pre-incubated with increasing concentrations of the inhibitor RNAs. In order to determine if Cas9 is functional, we performed cleavage assays in the absence and presence of small RNA inhibitors. A control experiment without an inhibitor showed that Cas9 efficiently cleaves the target (Figure 5.1b). However, the addition of the inhibitor at increasing ratios of 1:1; 1:2; 1:5; 1:10 (to sgRNA) showed decreasing cleavage of target DNA (Figure 5.1b). Inhibitor 1 was found to progressively inhibit cleavage as the ratio of inhibitor to sgRNA was increased. Inhibitors 2 and 3 completely abolished cleavage even at the lowest ratios (Figure 5.1 b). In order to test whether the inhibitors can stably bind to the sgRNA, we performed an electrophoretic mobility shift assay (EMSA).

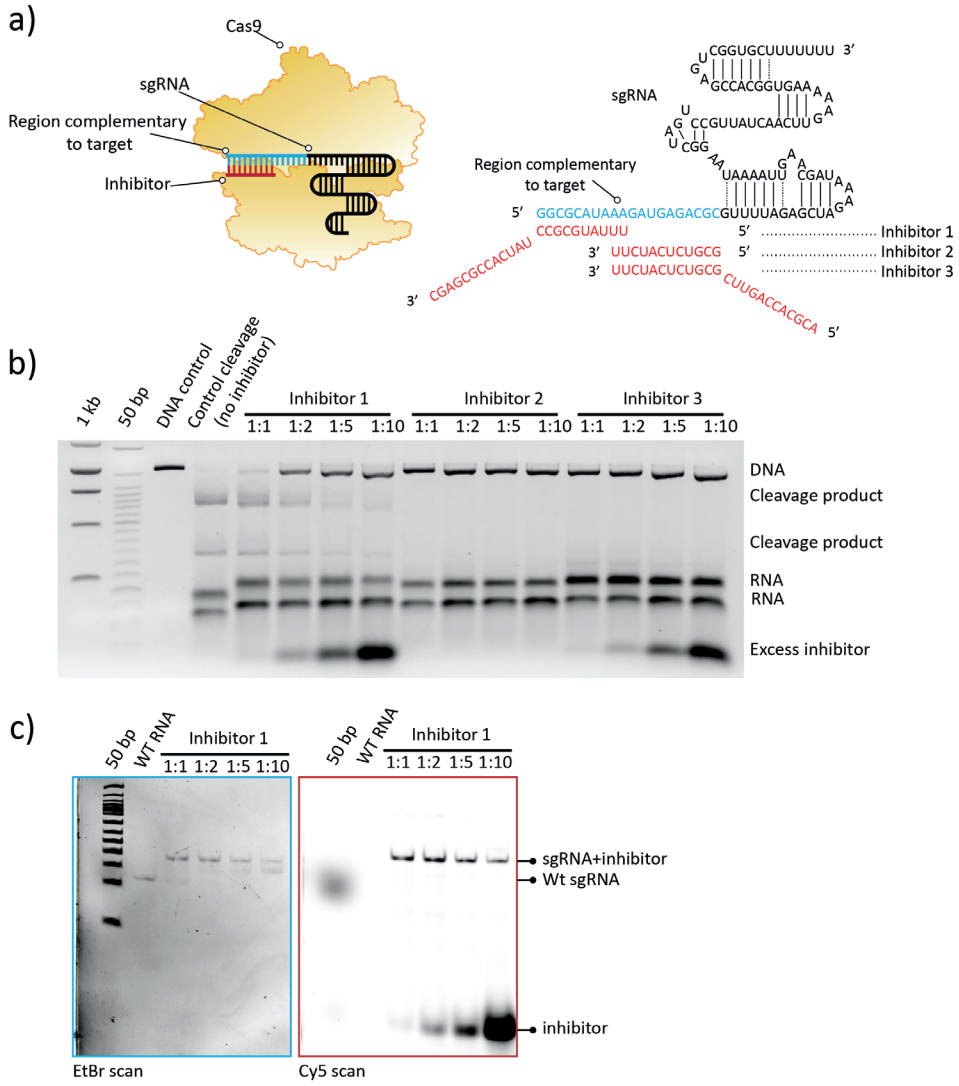

Figure 5.1: Small RNAs inhibit Cas9 cleavage in vitro. a) A schematic representation of the inhibitor molecules and Cas9. b) A 3% agarose gel showing an in vitro cleavage assay demonstrating the inhibition of cleavage. c) Electrophoretic mobility shift assay resolved using 10% PAGE showing that inhibitor 1 efficiently hybridizes with sgRNA.

The shift assay demonstrated that the inhibitors were stably hybridized to the sgRNA and shifted up in the gel, which suggested that the hybrid might be loaded by Cas9 (Figure 5.1 c, Supplementary Figure 5.1a, b).

In order to further elucidate the mechanism by which the small RNA molecules inhibit the catalytic activity of Cas9, we developed a single-molecule fluorescence assay in which the Cas9 protein, pre-incubated with the sgRNA and the Cy5-labeled inhibitor, is immobilized on the surface of the microscope slide (Figure 5.2a). After directly exciting the Cy5 fluorophores, we observe 1229 +/- 100 molecules per field of view on average for inhibitor 1, 1289+/- 84 for inhibitor 2 and 1247 +/- 71 for inhibitor 3, indicating that Cas9 efficiently loads the sgRNA-inhibitor hybrid (Figure 5.2b). A control experiment where Cas9 was pre-incubated only with the inhibitor, but without sgRNA showed only around 100 molecules per field of view on average for each inhibitor (Figure 5.2b), indicating that the observed high density of the molecules in the previous cases are indeed Cas9:sgRNA:inhibitor complexes. In order to monitor the interactions between the complexes immobilized on the surface and a DNA target, a Cy3-labelled target DNA was added and the interactions were monitored for 60 minutes by taking short snapshots over multiple fields of view every 10 minutes. Snapshot experiments were chosen in order to minimize the photobleaching of the fluorophores. The molecules were simultaneously excited by a 532 nm and a 633 nm laser in order to monitor the behaviour of Cy3-labeled target, and Cy-5 labeled Cas9:sgRNA:inhibitor complexes independently. A control, where no target has been added, showed that the inhibitor remained stably bound over the imaging time (Figure 5.2c, Supplementary figure 5.2a, b) with the number of molecules on the surface decreasing on average by 15 +/-5 percent due to photobleaching. Interestingly, we observed similar levels of loss of Cy5 fluorescence in the microfluidic chamber where the target DNA was added, indicating that the presence of the target did not affect the Cas9:RNA:inhibitor complex (Figure 5.2c, Supplementary Figure 5.2a, b). Incidently, we observed very few molecules appearing in the Cy3 channel, which indicated that the Cas9:sgRNA:inhibitor complexes did not bind to the target. No transient interactions were observed, which suggested that Cas9 did not interact with neither target nor PAM sequences when loaded with the sgRNA:inhibitor duplex. A control, where no inhibitor was hybridized to the sgRNA, showed fast and efficient binding (Figure 5.2d), demonstrating that the Cas9 protein is active. This indicates that the small RNA molecules inhibit the catalytic activity of Cas9 by preventing it from interacting with the target DNA.

To test whether the inhibitor molecule could be removed from the protein, we designed a DNA strand which is complementary to inhibitors 1 and 3, and may trigger the release of the inhibitor from the complex by competing with the base-pairing between the inhibitor and the sgRNA (Figure 5.2e). Time-lapse images over 60 minutes in the microfluidic chamber, where the trigger and the target were added together, showed a very rapid loss of Cy5 fluorescence, where the number of fluorescent molecules decreased by 50% over the first 10 minutes with the number of molecules on the surface decreasing more slowly over the next 50 minutes with 30% of the Cy5-labeled molecules remaining after 60 minutes.

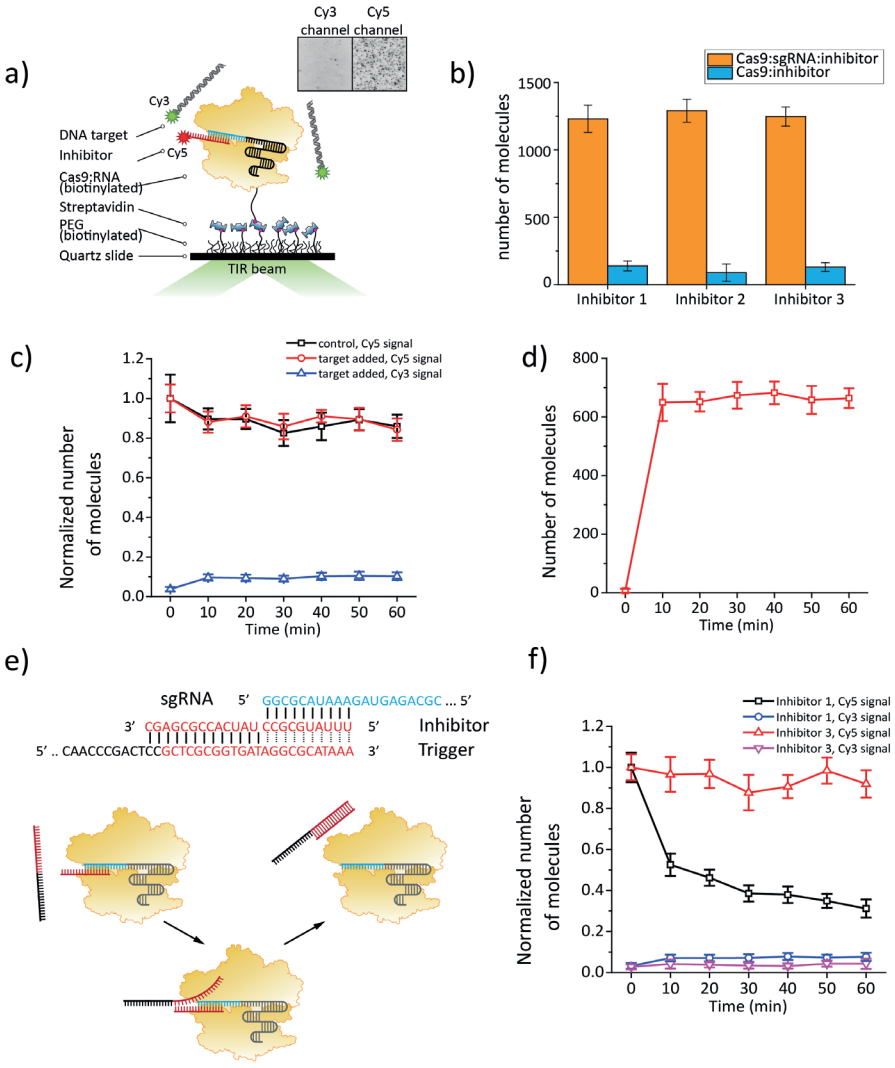

Figure 5.2: Single molecule fluorescence reveals the mechanism of inhibiton. a) schematic representation of the single-molecule assay b) a histogram showing the average number of molecules per field of view. The molecules were counted as fluorescent spots in the Cy5 channel. The average was taken over 15 snapshots over different fields of view. The error bars represent standard deviation. The data is the average of three independent experiements taken over three different days c) scatterplot showing number of molecules on the surface over 60 minutes for inhibitor 1. The molecules were counted as fluorescent spots in the Cy5 channel for inhibitor and the cy3 channel for target. The number of molecules was normalized by the number of molecules on the surface at time= 0. The average was taken over 15 snapshots over different fields of view per time point. The error bars represent standard deviation. The data is the average of three independent experiments taken over three different days. d) a scatter plot showing the binding of DNA target to Cas9:sgRNA complexes on the microscope slide. Cy3-labeled DNA was imaged. The average was taken over 15 snapshots over different fields of view. The error bars represent standard deviation. The data is the average of three independent experi-

ments taken over three different days. e) a schematic representation of the assay with the addition of the trigger molecule f) a scatter plot showing the number of molecules on the surface after the addition of the trigger molecule in combination with the target. the number of molecules was normalized by the number of molecules on the surface at time= 0. The average was taken over 15 snapshots over different fields of view. Inhibitor molecules were counted as fluorescent spots in the Cy5 channel and target molecules as fluorescent spots in the Cy3 channel. The error bars represent standard deviation. The data is the average of three independent experiments taken over three different days.

This was observed in the case of inhibitor 1, but not inhibitor 3 (Figure 5.2f). The behavior of the complexes with inhibitor 3 when the trigger was present in the channel was observed to be identical to the control when neither trigger nor target was added to the channel (Figure 5.2f, Supplementary Figure 5.2b). As the extent of base-pairing between Inhibitor 1 and guide is 2nt shorter than in the case of Inhibitor 3, we set out to determine, whether it is the extent of base-pairing between sgRNA and the inhibitor RNA or the position of the latter with respect to the sgRNA, which gives rise to the loss of fluorescence. We designed two inhibitor molecules, Inhibitor 4 which has a 2nt longer base-paired region than Inhibitor 1, and inhibitor 5 which has a 2nt shorter base-paired region than Inhibitor 3 (Supplementary Figure 5.2c). After performing the same experiments with the trigger molecules, we observed loss of fluorescence in the case of Inhibitor 4, identical to that of Inhibitor 1 (Supplementary Figure 5.2d). Inhibitor 5 did not show any loss of florescence, similar to Inhibitor 3. This shows that the trigger is only able to induce the release of an inhibitor only when it is situated at the end of the sgRNA, suggesting that the protein holds onto the inhibitor more strongly when it is hybridized to the seed region of the sgRNA. This may explain why the cleavage inhibition is less efficient in the case of inhibitor 1 (Figure 5.1b). Interestingly, despite the loss of Cy5 fluorescence indicating the release of inhibitor 1, a correlating appearance of molecules in the Cy3 channel was not observed. This suggests, that despite the inhibitor leaving the protein, the protein remains locked in an inactive conformation, unable to further interact with the target.In order to further confirm that Cas9 strongly binds the sgRNA:inhibitor duplex we have attempted to perform an electrophoretic mobility shift assay. However, with no Cas9-bound DNA present, Cas9:RNA ribonucleoprotein complexes did not enter the gel. In addition, we have performed atomic force microscopy (AFM) imaging of the assembled RNPs in order to observe whether the inhibitor induces a substantial conformational change (data not shown). Unfortunately, we could not see any clear differences between the RNPs with and without inhibitor or any clear structural features as Cas9 is a small and globular protein. Therefore, in order to observe any structural intermediates, a technique such as cryo electron microscopy or X-ray crystallography should be used. Currently, we are performing in vivo plasmid loss assays to see whether the inhibition also takes place in living cells. Furthermore, an in vitro experiment with purified miRNA from HEK297T cells which could inhibit cleavage is under consideration, as a mimic of an in vivo condition.

5.3 Discussion

Despite the enormous interest in Cas9 and its potential in therapeutics, regulating its activity remains challenging in areas where precise control of the process is required. Moreover, if Cas9 is to be applied in combination with another RNA-guided protein, programmed to target a similar sequence, the interactions between the RNAs may potentially give rise to unexpected and unwanted effects. In addition, it has been demonstrated that sgRNA molecules that form hairpin structures at the PAM-distal region can increase the specificity of Cas9 by making it energetically unfavourable for it to pair with off-target sites, which decreases the overall activity of Cas9[17]. We show that Cas9 cleavage of the target DNA can be efficiently inhibited by small RNA molecules which are hybridized to the sgRNA and loaded into Cas9. Using single-molecule fluorescence, we have demonstrated that the sgRNA:inhibitor hybrids are efficiently loaded into Cas9 and remain stable over at least 60 minutes. This suggests that the negatively charged groove between the REC and the Nuclease lobes of Cas9 can accommodate an RNA duplex without the necessity for structural rearrangements upon PAM recognition, which are necessary to accommodate the target DNA[18]. Furthermore, we did not observe interactions with the target DNA eventhough a control experiment with bare RNA showed efficient target binding, which demonstrates that small RNA inhibits the function of Cas9 by preventing it from interacting with the target. A trigger molecule complementary to the inhibitor was observed to be able to trigger the release of the inhibitor from the RNP. Surprisingly, no target binding was observed even after the inhibitor molecule has been removed, which further implies that the loading of inhibitor:sgRNA hybrid causes Cas9 to adopt an inactive conformation which it cannot change even when the inhibitor is no longer present. Interestingly, we did not observe the loss of fluorescence indicative of the inhibitor dissociating from the complex with inhibitor 3, which suggests that the protein interacts with it more strongly when it is placed in the seed region as the electrophoretic mobility shift assays showed that all sgRNA molecules hybridize to the inhibitors equally efficiently. In addition, a recent study demonstrated that riboregulated toehold-gated gRNA can be used to control Cas9 function by the addition of a trigger molecule which unfolds the toehold-gated gRNA[19]. Our work suggests that this is only possible before Cas9 is loaded with a hybridized or folded RNA molecule. Therefore, our findings demonstrate that delivery of DNA or sgRNA and mRNA for translating Cas9 and assembling the RNP complexes in vivo may be susceptible to unwanted effects such as possible inhibition by endogenous RNA molecules. However, further research will be necessary to elucidate the extent of this inhibition in vivo. Nevertheless, electroporating assembled RNP complexes into cells may be the strategy that will suffer the least from unexpected effects.

5.4 Materials and methods

sgRNA production

To make the sgRNA, we first PCR amplify a dsDNA template, which contains the target sequence from a DNA plasmid (pgRNA-bacteria plasmid from Addgen), using a primer that contains a T7 promoter. The following thermal cycling conditions were used to generate the PCR template: 98°C for 3 minutes; 98°C for 10 seconds; 65°C for 20 seconds; 72°C for 15 seconds; go to step 2 for 29 cycles and 72°C for 8 minutes. The PCR template was verified using gel electrophoresis (1,5% agarose, 1X TBE buffer, 120V for 90 minutes) and subsequently purified using the WizardSV Gel and PCR Clean-Up System (Promega) according to the manufacturer's instructions. A single gRNA was then transcribed from the PCR template using the RiboMaxTM Large Scale RNA Production Systems kit (Promega) according to the manufacturer's instructions. Following transcription, RNA products were purified using the RNeasy MinElute Cleanup Kit (Qiagen) according to the manufacturer's instructions. RNA quality was verified using gel electrophoresis (Mini-Protean TBE-Urea Precast Gels (Bio-Rad), 200V for 30 minutes). Gels were visualized under UV light in a Biorad ChemiDOCT MP imaging system.

Cleavage assays

For cleavage assays, sgRNA was prepared by heating up to 95°C for 10 minutes and slowly cooling down (1°C every 4 minutes until a final temperature of 4°C). Cas9 was pre-incubated with sgRNA in a 1X Cas9 Nuclease Reaction Buffer (New England Biolabs) in a molar ratio of 1:10 for 30 minutes at 25°C. Thereafter, target DNA was added in a molar ratio of 1:10 to Cas9, effecting a total molar ratio of 100:10:1 of sgRNA:dCas9:DNA. An excess ratio was used to ensure complete cleavage. The complex was incubated for 30 minutes at 37°C. Proteinase K was then used to digest Cas9 for 15 minutes at 37°C. Cleavage products were verified using gel electrophoresis (1,5% agarose, 1X TBE buffer, 120V for 90 minutes). Gels were visualized under UV light in a Biorad ChemiDOCT MP imaging system.

Electrophoretic mobility shift assay (EMSA)

For EMSA, sgRNA was incubated with different concentrations of inhibitor RNA (molar ratios of (1:1;1:2;1:5;1:10) by heating up to 95°C for 10 minutes and slowly cooling down (1°C every 4 minutes until a final temperature of 4°C). the sgRNA-inhibitor hybrids were analyzed using gel electrophoresis (10% 1X TBE-Precast Gels (Invitrogen), 90V for 90 minutes).

Recombinant SpCas9 purification

The pET plasmid encoding (6x)His-tagged Cas9 was transformed into BL21 (DE3), Rosetta. Transformed bacterial cells were moved to a 400ml of fresh LB medium containing 50ug/ml kanamycin. Incubate the culture with shaking (200rpm) at 18°C for 24 hours. Optical density was monitored and Cas9 protein expression was induced (A550=0.6) by using 0.5mM IPTG at 18C for 24hours. After the cells were harvested by centrifugation (5000xg) for 10minutes (at 4°C), bacterial cells were resuspended with lysis buffer [20 mM Tris–HCl (pH 8.0), 400mM NaCl, 10mM b-mercaptoethanol, 1% Triton X-100, 50mg aprotinin, 50mg antipain, 50mg bestatin, 1mM PMSF (phenylmethylsulfonyl fluoride)] (Sigma-Aldrich) and sonicated on ice. The lysate was centrifuged at 6000 rcf for 10min(4°C) and supernatant solution was mixed with 2ml of Ni-NTA slurry (Qiagen) at 4°C for 1 and half hour. The lysate/Ni-NTA mixture was loaded onto a column (Biorad) with capped bottom outlet. Loaded sample was washed multiple times with pre-made wash buffer [20 mM Tris–HCl (pH 8.0), 400mM NaCl, 10mM b-mercaptoethanol] and (6x)His-tagged SpCas9 was eluted with Elution Buffer [20 mM Tris–HCl (pH 8.0), 400mM NaCl, 10mM b-mercaptoethanol, 200mM Imidazole]. Finally, buffer containing eluted SpCas9 protein was changed to storage buffer [10mM HEPES-KOH (pH 7.5), 250mM KCl, 1mM MgCl2, 0.1mM EDTA, 7mM b-mercaptoethanol and 20% glycerol] by using centrifugal filter (Amicon Ultra 100K). The purified SpCas9 protein was frozen with liquid nitrogen and stored at -80°C.

Biotinylation of the recombinant SpCas9

The process of linking biotin to the recombinant protein was carried out in-vitro and proceeded during the process of protein purification. After loading the SpCas9 over-expressed bacterial lysate and Ni-NTA mixture onto a column (Biorad), mixed sample was washed multiple times with wash buffer [20 mM Tris–HCl (pH 8.0), 400mM NaCl]. Then we added 10-fold molar excess of maleimide-biotin (Sigma-Aldrich) to SpCas9 solution and incubate for overnight at 4°C (mix gently with rotator). To get rid of unbound maleimide-biotin chemicals, mixed sample was washed sufficiently with wash buffer [20 mM Tris–HCl (pH 8.0), 400mM NaCl]. Finally, biotinylated SpCas9 protein was eluted with elution Buffer [20 mM Tris–HCl (pH 8.0), 400mM NaCl, 200mM Imidazole], then the protein concentration was measured by spectrophotometer (Nanodrop 2000, Thermo Fisher Scientific). Eluted SpCas9 protein was further purified with size exclusion chromatography. The biotinylation degree of the wild-type SpCas9 protein was calculated with commercial kit (Pierce) and it reached about 100% for two Cysteine sites (Cys80/Cys574). Biotinylated SpCas9 protein was stored in storage buffer [10mM HEPES-KOH (pH 7.5), 250mM KCl, 1mM MgCl2, 0.1mM EDTA, 7mM b-mercaptoethanol and 20% glycerol] and purified protein was frozen in liquid nitrogen and store at -80°C.

Single-molecule two-color FRET

Single-molecule fluorescence measurements were performed with a prism-type total internal reflection fluorescence microscope. 0.1mg/ml Streptavidin was added to a polyethylene glycol-coated quartz surface and incubated for 2 minutes before being washed with T50 (10 mM Tris-HCl (pH 8.0), 50 mM NaCl). Biotinylated Cas9 was pre-incubated with sgRNA and Cy5-labeled inhibitor RNA (ratio 1:2:10) at 37 degrees for 20 minutes in NEB buffer 3 (100 mM NaCl, 50 mM Tris-HCl, 10 mM MgCl2, 1 mM DTT) and then added to the chamber containing Streptavidin. After 2 minutes of incubation unbound Cas9 and RNA molecules were washed away with an imaging buffer (50mM HEPES-NaOH [pH7.5], 150mM NaCl, 2mM MgCl2, 1% glucose (Dextrose monohydrate), 1mM Trolox (2.5mg/10ml), 1mg/ml glucose oxydase [Sigma], 170ug/ml catalase [Merck]). 8 nM Cy3 labeled DNA substrate in imaging buffer was added to the channel. For the experiments with the trigger, 400nM of unlabeled trigger was added in addition to 8nM of Cy-3 labeled DNA. Simultaneous laser excitation (532 nm and 633nm) was used. Following the addition of DNA, 15 snapshots of different fields of view consisting of about 10 frames were collected every 10 minutes for a total of one hour. Fluorescence signals of Cy3 and Cy5 were collected through a 60× water immersion objective (UplanSApo, Olympus) with an inverted microscope (IX73, Olympus). The 532 nm laser scattering was blocked out by a 532 nm long pass filter (LPD01-532RU-25, Semrock). The Cy3 and Cy5 signals were separated with a dichroic mirror (635 dcxr, Chroma) and imaged using an EM-CCD camera (iXon Ultra, DU-897U-CS0-#BV, Andor Technology).

Data acquisition and analysis

Using a custom-made program written in Visual C++ (Microsoft), a series of CCD images of time resolution 0.1s was recorded. The time traces were extracted from the CCD image series using IDL (ITT Visual Information Solution) employing an algorithm that looked for fluorescence spots with a defined Gaussian profile and with signals above the average of the background signals. Colocalization between Cy3 and Cy5 signals was carried out with a custom-made mapping algorithm written in IDL. The extracted time traces were processed using Matlab (MathWorks) and Origin (Origin Lab).

5.5 Supplementary Information

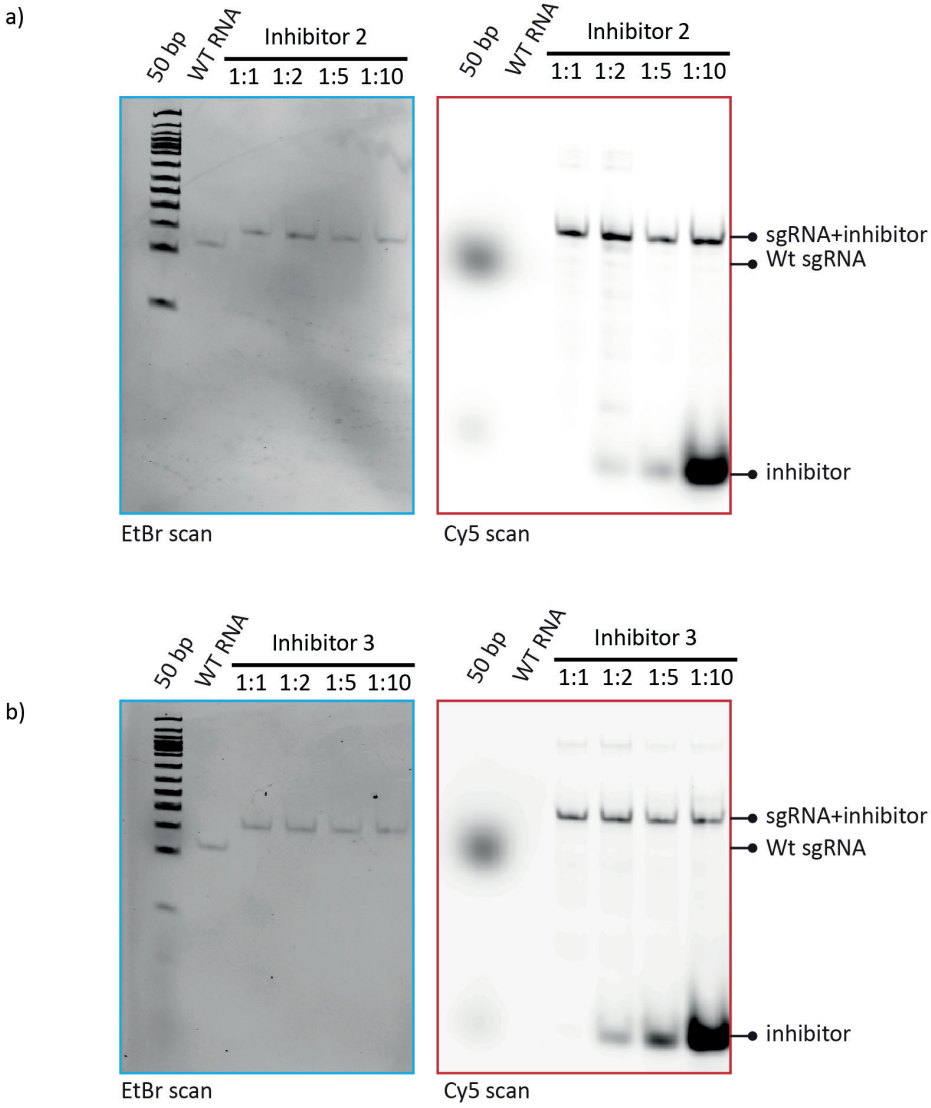

Figure S5.1: Inhibitors efficiently hybridize with sgRNA. a) Electrophoretic mobility shift assay resolved using 10% PAGE showing that inhibitor 2 efficiently hybridizes with sgRNA. b) Electrophoretic mobility shift assay resolved using 10% PAGE showing that inhibitor 3 efficiently hybridizes with sgRNA.

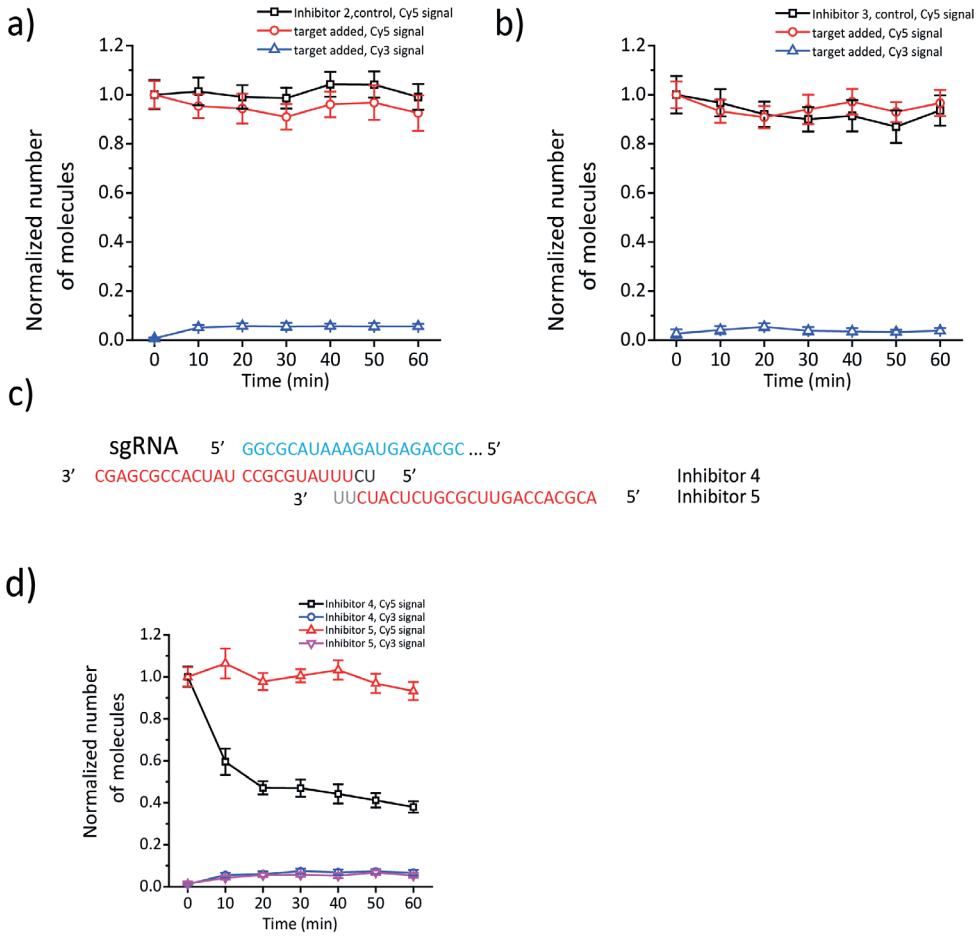

Figure S5.2: Behavior of Cas9:sgRNA:inhibitor complexes over 60 minutes. a) a scatter plot showing the number of molecules on the surface over time for inhibitor 2. b) a scatter plot showing the number of molecules on the surface for inhibitor 3. In both cases The number of molecules was normalized by the number of molecules on the surface at time= 0. The average was taken over 15 snapshots over different fields of view. The error bars represent standard deviation. The data is the average of three independent experiments taken over three different days. c) Schematic showing Inhibitors 4 and 5. d) a scatter plot showing the number of molecules on the surface after the addition of the trigger molecule in combination with the target for Inhibitors 4 and 5. the number of molecules was normalized by the number of molecules on the surface at time= 0. The average was taken over 15 snapshots over different fields of view. Inhibitor molecules were counted as fluorescent spots in the Cy5 channel and target molecules as fluorescent spots in the Cy3 channel. The error bars represent standard deviation. The data is the average of three independent experiments taken over three different days.

Supplementary tables

Table S5.1: RNA sequences used in this study

sgRNA	5'GGCGCAUAAAGAUGAGACGCGUUUUAGAGCUAGAAAUAGCAAG UUAAAAUAA-GGC UAGUCCGUUAUCAACUUGAAAAAGUGGCACCGA GUCGGUGCUUUUUUU 3'
Inhibitor 1	5' UUUAUGCGCCUAUGCGCCUGUUGCC 3'-**Cy5**
Inhibitor 2	5' GCGUCUCAUCUU 3'- **Cy5**
Inhibitor 3	5' ACGCACCAGUUCGCGUCUCAUCUU 3'- **Cy5**
Inhibitor 4	5' UUUAUGCGCCUGUUGCC 3'- **Cy5**
Inhibitor 5	5' ACGCACCAGUUCGCGUCUCAUC 3'- **Cy5**

Inhibitor molecules were ordered labeled with a Cy5 dye on the 3' end.

Table S5.1: DNA sequences used in this study

PCR template FWD	5' TAATACGACTCACTATAGGTACGGTTATCCACAGAATCAGTTTTAG AGCT AGAA-ATAGCAAGTTAAAATAAGG 3'
PCR template REV	5' AAAAAAAGCACCGACTCGGTGCCAC 3'
Non-target strand	5' CCAGCTGTCTGCACAGGAGAAATCCCTGCT**GGCGCATAAAGAT GAGACGCTG-G**AGTACAAACGCCAGCTGGCTGCACTTGGCGACAA GGTTACGTATCAG 3'
Target strand	5' CTGATACGTAACCTTGTCGCCAAGTGCAGCCAGCTGGCGTTTGT ACTCCAG **CGTCTCATCTTTATGCGCC**AGCAGGGATTTCTCCTGTGC AGACAGCTGG 3'-**Cy3**
Trigger 1	5' GCTCGCGGTGATAGGCGCATAAACGACTCCCATCCCGCCCAACC CGACT **CCGC-TCGCGGTGATAGGCGCATAAA** 3'
Trigger 3	5' AAGATGAGACGCGAACTGGTGCGTCGACTCCCATCCCGCCCAA CCCGACTCC **AAGATGAGACGCGAACTGGTGCGT** 3'

Target regions are shown in bold, PAM sequences are shown in red. The target strand has been ordered with a Cy3 fluorophore at the 3' end. PCR templates were used to produce sgRNA. In Triggers 1 and 3, the region complementary to Inhibitors 1 and 3 respectively are shown in blue.

References

1. Barrangou, R., et al., CRISPR provides acquired resistance against viruses in prokaryotes. Science, 2007. 315(5819): p. 1709-12.
2. Marraffini, L.A., CRISPR-Cas immunity in prokaryotes. Nature, 2015. 526(7571): p. 55-61.
3. Makarova, K.S., et al., A putative RNA-interference-based immune system in prokaryotes: computational analysis of the predicted enzymatic machinery, functional analogies with eukaryotic RNAi, and hypothetical mechanisms of action. Biol Direct, 2006. 1: p. 7.
4. Mohanraju, P., et al., Diverse evolutionary roots and mechanistic variations of the CRISPR-Cas systems. Science, 2016. 353(6299): p. aad5147.
5. Wiedenheft, B., S.H. Sternberg, and J.A. Doudna, RNA-guided genetic silencing systems in bacteria and archaea. Nature, 2012. 482(7385): p. 331-8.
6. Jinek, M., et al., A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science, 2012. 337(6096): p. 816-21.
7. Gasiunas, G., et al., Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. Proc Natl Acad Sci U S A, 2012. 109(39): p. E2579-86.
8. Sapranauskas, R., et al., The Streptococcus thermophilus CRISPR/Cas system provides immunity in Escherichia coli. Nucleic Acids Res, 2011. 39(21): p. 9275-82.
9. Doudna, J.A. and E. Charpentier, Genome editing. The new frontier of genome engineering with CRISPR-Cas9. Science, 2014. 346(6213): p. 1258096.
10. Yang, H., et al., One-step generation of mice carrying reporter and conditional alleles by CRISPR/Cas-mediated genome engineering. Cell, 2013. 154(6): p. 1370-9.
11. Chew, W.L., et al., A multifunctional AAV-CRISPR-Cas9 and its host response. Nat Methods, 2016. 13(10): p. 868-74.
12. Shalem, O., et al., Genome-scale CRISPR-Cas9 knockout screening in human cells. Science, 2014. 343(6166): p. 84-87.
13. Heckl, D., et al., Generation of mouse models of myeloid malignancy with combinatorial genetic lesions using CRISPR-Cas9 genome editing. Nat Biotechnol, 2014. 32(9): p. 941-6.
14. Paquet, D., et al., Efficient introduction of specific homozygous and heterozygous mutations using CRISPR/Cas9. Nature, 2016. 533(7601): p. 125-9.
15. Wang, T., et al., Genetic screens in human cells using the CRISPR-Cas9 system. Science, 2014. 343(6166): p. 80-4.
16. Zhao, N., et al., Combinatorial CRISPR-Cas9 and RNA Interference Attack on HIV-1 DNA and RNA Can Lead to Cross-Resistance. Antimicrob Agents Chemother, 2017. 61(12).
17. Kocak, D.D., et al., Increasing the specificity of CRISPR systems with engineered RNA secondary structures. Nat Biotechnol, 2019. 37(6): p. 657-666.
18. Nishimasu, H., et al., Crystal structure of Cas9 in complex with guide RNA and target DNA. Cell, 2014. 156(5): p. 935-49.
19. Siu, K.H. and W. Chen, Riboregulated toehold-gated gRNA for programmable CRISPR-Cas9 function. Nat Chem Biol, 2019. 15(3): p. 217-220.

6

Concluding remarks

Here, I sketch a perspective on the challenges that are associated with developing novel point-of-care diagnostic tests for resource-limited settings. I discuss the adaptable potential of the DNA-detection scheme, that we developed in chapter 4, and provide a few concluding remarks on the barriers to diagnosing infectious diseases, such as visceral leishmaniasis, in resource-limited settings.

6.1 Diagnosing infectious diseases in resource-limited settings

The current discussions on testing and tracing during outbreaks such as the 2020 COVID-19 pandemic and emerging infectious outbreaks such as the Zika and Ebola epidemics, demonstrate that diagnostics is at the forefront of global healthcare to guide treatment, surveillance, and containment efforts[1]. In general, diagnostic instruments are expensive, require skilled personnel, a well-equipped laboratory, and basic infrastructure to operate. One could easily imagine that these resources are readily available in high-resource settings, and that diagnostics thus would not be hindered. However, the current COVID-19 pandemic demonstrates that large-scale diagnostics are still limited in practise, even in high-resource settings. Diagnostic challenges are even more confounded in resource-limited settings due to a lack of infrastructure, equipment, skilled-users, and funding for reagents[2]. Thus, cost-effective and field-deployable point-of-care (PoC) diagnostic tests would greatly improve diagnostics, especially in resource-limited settings.

In order to reduce the use of empirical diagnosis (based on symptoms) in resource-limited settings, it is essential to improve diagnostic capabilities in order to detect infectious diseases during the early asymptomatic stage of the disease[3]. In the absence of adequate infrastructure in resource-limited settings, the role of reliable PoC tests would be revolutionary. More specifically, a reliable PoC test, that could serve as a confirmatory test, would benefit both the patients and the medical staff. The limited capacity (electrical supply, cold chain, reliable supply of diagnostic tests and medication) in endemic resource-limited settings poses a major obstacle in disease management. To understand such obstacles, we explored the different levels of the healthcare system during our field research (chapter 3) (Figure 6.1).

Level 1 (base of the pyramid) represents a very basic homestead setting, wherein users do not have any medical experience. This diagnostic setting would maximally allow for a home pregnancy test wherein the user can follow simple instructions to perform the test themselves. Level 2 represents a basic setting where, in contrast to level 1, users are minimally trained and have access to basic resources such as medical gloves. As discussed in chapter 3, community healthcare workers (who are often well-respected community members such as teachers and religious pastors) could potentially perform PoC diagnostics at this level 2. Level 3 represents a well-equipped setting, such as a reference laboratory. During our field research, we observed that that reference laboratories had the capacity to perform direct agglutination tests (DATs) that are widely used for serodiagnosis of visceral leishmaniasis (VL), based on antigen-antibody reactions[4]. Briefly, promastigotes that are pretreated with trypsin, stained, and preserved in formalin are used as an antigen that shows agglutination (clumping of particles) when antibodies against leishmania parasites are present in a patient's serum or whole-blood sample. Although the test

is performed at room temperature, the antigen needs to be stored in a refrigerator. The DAT requires a trained person to perform the serial dilutions of the antigen. The preparation incubation time is at least 8 hours, while the antigen-antibody reaction time is approximately 18 hours[5]. Nonetheless, DATs are routinely performed in Kenya and Uganda (Figure 6.2). The major limitation of a DAT is that it cannot be used as a test-of-cure. Level 4 represents an advanced setting such as a hospital wherein there are doctors, and advanced surgical procedures can be done (Figure 6.1).

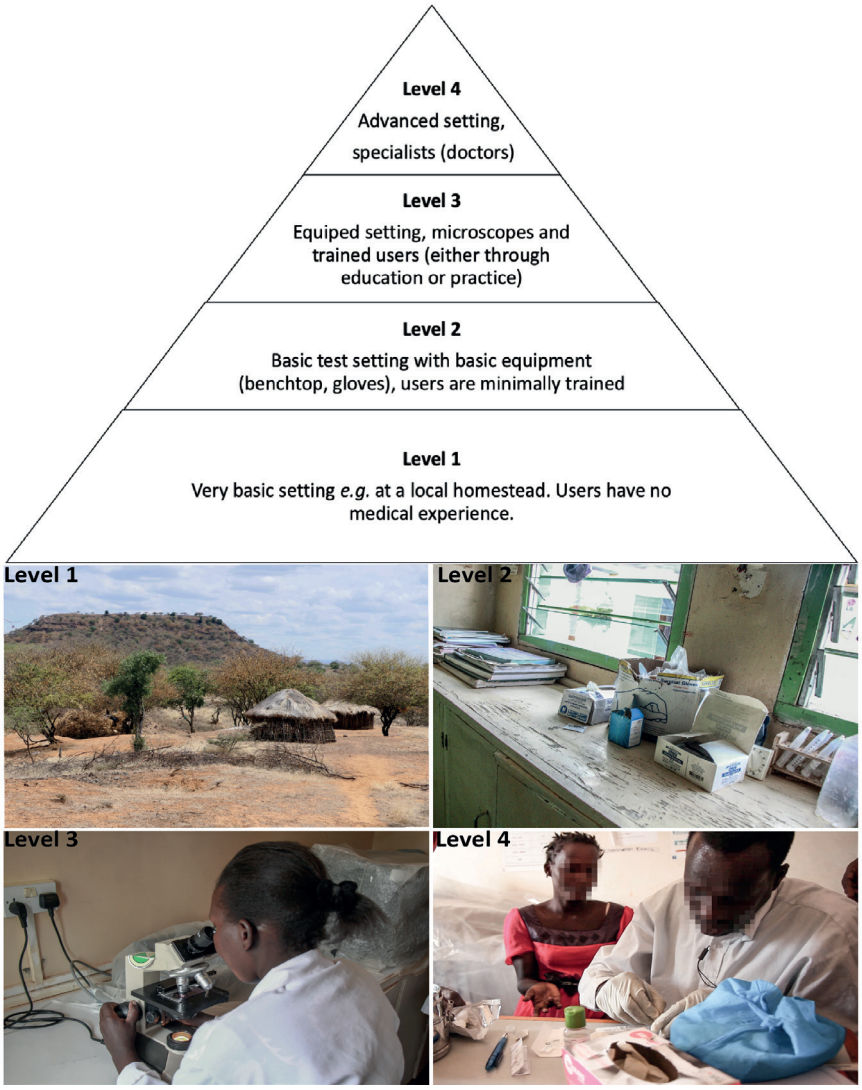

Figure 6.1: Different levels of the healthcare system in Kenya (top panel) that represents different diagnostic settings in Kenya (bottom panel). Photo credit: Astrid ten Bosch and Cees Dekker.

Figure 6.2: Direct agglutination test (DAT) (left panel) in a central laboratory at level 3 of the Kenyan healthcare system (right panel). Photo credit: Astrid ten Bosch and Cees Dekker.

Taking this capacity into consideration, the DNA-based detection scheme that we have developed in this study (chapter 4) could be extremely valuable in these resource-limited settings, most likely at level 3, as it could potentially serve as a test-of-cure test. One major limitation of our DNA-based detection scheme is the use of hydrogen peroxide for the final colorimetric read-out, which currently needs to be kept refrigerated. This limitation may be circumvented by using stable forms of hydrogen peroxide at room temperature, of which there are commercial companies that may be willing to license their technology for this purpose (agreements on intellectual property (IP) rights have to be made). Another solution may be to use closed microfluidic devices that keep the hydrogen peroxide stable, although testing and optimization need to be performed to address this solution.

Nucleic-acid-based detection methods are extremely valuable as they are independent of the patients' immune response, and they can serve as a test-of-cure. Polymerase chain reaction (PCR), that was invented in 1984, was a revolutionary discovery that has paved the way for the field of molecular biology. It is still the most sensitive and specific DNA detection method used, but it requires sample preparation, equipment, and trained users. Similarly, DNA sequencing, which lead to the human genome project in 2003, is a very powerful tool (e.g. the MinION sequencer), and in many years to come, it may be used in resource-limited settings for diagnosis. However, DNA sequencing is currently hindered by sample preparation and costs. Although numerous DNA detection methods exist, there are clear performance trade-offs between specificity and sensitivity, and the need for complex equipment, basic infrastructure such as a stable source of electricity, and skilled users. Nucleic-acid-based detection methods are more accurate and reliable than immunological methods but they need further validation for PoC use.

The discovery of CRISPR-Cas systems has revolutionized applications such as genome engineering and molecular diagnosis. The power of CRISPR-Cas technologies for diagnostics is its ease of programmability. More specifically, CRISPR-Cas9 has been extensively studied worldwide, with a plethora of information that is available regarding its crystal structure[6], allosteric assembly[7], and target search mechanisms[8]. Indeed, researchers have explored the use of RNA-guided CRISPR-Cas systems for diagnostics (see chapter 1; table 1.4), which have been typically applied in molecular biology to target and cleave specific nucleic acids for genome editing. Given the combination of its simplicity and ease of programmability, and direct binding to double-stranded DNA, it seems intuitive that Cas9 is readily used for diagnostic applications such as pathogen detection (as we have done in chapter 4). The most prominent example of the use of CRISPR-Cas systems for diagnostics is the SHERLOCK detection platform[9]. The first SHERLOCK detection platform relied on the use of fluorescence for the readout, which is suitable for high-resource environments, yet unsuitable for resource-limited settings. Hence, the SHERLOCK researchers have built on this foundation and developed version 2 which is a multiplexed assay with a lateral flow assay readout[10]. Notably, DETECTR[11] is also a well-known CRISPR-based diagnostic platform, and Tsou et al coupled the DETECTR assay with a lateral flow assay (LFA) readout[12]. When comparing SHERLOCK to DETECTR, SHERLOCK requires an additional reverse transcription reaction, and thus DETECTR is more practical than SHERLOCK. Despite their interest, all these CRISPR-based biosensing tools need to be validated with large sample sizes and tested in the field[12].

Future prospects for CRISPR-Cas-based diagnostics are very encouraging. Yet, it is well documented that CRISPR-Cas9 has off-target effects, which can be very problematic for diagnostics. Although there are strategies to mitigate off-target effects, they are far from perfect. To alleviate possible off-target effects, we employed a dual detection system in our diagnostic scheme, wherein recombinase polymerase amplification (RPA) provides the first line of specificity and dCas9 provides the second and final specificity check (chapter 4). In this way, we circumvented the detection of non-specific amplicons from the RPA reaction, and provide dCas9 with the relevant amplified pathogenic-DNA targets among a vast background of genomic DNA. Our dual target recognition (RPA and dCas9) and dual isothermal amplification (RPA and rolling circle amplification (RCA)) approach enhances both the specificity and sensitivity of our detection scheme.

6.2 Future research directions

Progressing from a proof-of-principle study in a laboratory to a field-deployable test is a complex and tedious process. While our research so far focussed on a proof-of-principle demonstration in the lab, there is still a long road ahead, from implementing tests in a certain packaging format to field tests[13]. There are many

different options for the format of a PoC test: lateral flow assays (LFA) (paper-based systems); microfluidic systems (lab-on-a-chip fluidic circuits); electrical sensors; smartphone-based approaches; or combinations thereof. The diagnostic setting (i.e., the particular location and environment where the diagnostic test will be used) is crucial for the technology and its commercial use and success. PoC testing can range widely in its applications: from the bedside in the emergency room at a well-equipped hospital, to a central laboratory, a doctor's office, at home, in the field in resource-limited settings, in the pharmacy or in a local shop that also supplies medication. Determining when and where the test will be used will put constraints on the test format. We now describe lateral flow assays and microfluidic systems, and discuss what systems would fit best.

An LFA is a qualitative analytical method that detects a target analyte. LFAs rely on capillary forces (passive movement of liquid) of a sample over a paper-based membrane. Different reagents are immobilized on the membrane prior to use, that will react with the sample upon wetting. A standard LFA consists of a sample entry point (e.g. where a blood sample is placed); a conjugate pad that contains capture molecules such as antibodies that are conjugated to a colored molecule for the read-out; the reaction membrane (usually made of nitrocellulose) that contains the control line (for test validity) and the test line (specific to the analyte in question); and finally, the absorption pad that serves as a waste reservoir and ensures a uni-directional flow (i.e., no backflow)[14]. LFAs are very attractive for PoC diagnostics as they are cost effective, easy to use and transport, the results can be seen within minutes, and they are easy to dispose. The most widely used LFA is the urine-based pregnancy test. However, the simplicity of the paper-based format has associated drawbacks. For example, extra washing steps, and increased incubation time (e.g. ~30 minutes incubation for Cas9 to bind to the target DNA) cannot be facilitated in an LFA format (Table 6.1). LFAs are suitable for simple assays that do not require sample preparation, and that address samples with high analyte concentrations. LFAs are generally not suitable for multiplexing[15], and high throughput. Another limitation is that LFAs have a subjective readout ("is that really a positive line?"). Batch-to-batch variation might also require a standardized card with relative intensities for each batch.

When developing novel PoC diagnostic tests, one challenge for the developer is to reduce the complexity of use for the end user. In this manner, microfluidics is an enabling technology[16]. Microfluidic devices are a set of micro-channels that are moulded into a material, and are connected to each other to perform a desired task in a controlled and automated manner (mixing, sorting, incubating). The network of micro-channels serves as a "lab-on-a-chip" device that can automatically perform complex reactions in a single device[17].

Table 6.1: Pros and cons of lateral flow assays and microfluidic devices[14,27,28].

	Lateral flow assays	Microfluidic devices
Pros	Rapid diagnostics at PoC Easy to use and transport Equipment free (electricity and cold chain not required) Compatible with all sample types Self-contained, important for hazardous waste (external components depend on the design) Long term storage (long shelf life) Single use to prevent cross-contamination Multiplexing possible	
	Overall low cost (mass production reduces costs) Easy disposal Can exclude plastic housing if only used for the read-out (reduce costs and waste – depends on the assay design) Decreased research and development time High commercialization potential	Relatively low cost (depends on fabrication and materials for mass production) Modular fabrication is possible (paper and plastic hybrid devices) Much smaller reagent volumes (can reduce and offset fabrication costs) Possibly quantitative (depends on the design and/ read-out) Washing and mixing/agitation steps possible (important for our design see chapter 4) Easier sample pre-treatment Increased reaction/incubation times for sequential reactions (important for our design see chapter 4) More enzyme activity upon rehydration
Cons	Material batch variability (e.g. variable plastic used in a hybrid microfluidic device; unless plastic is only used for external housing)	
	Generally, but not exclusively qualitative (binary) Sample pre-treatment (for viscous samples) is often required Limited reaction/incubation time due to capillary flow time (can be optimized/ improved using longer strips) Less controlled fluid movement (user/design dependent; can reduce precision) Capillary flow time variations between batches Restricted fabrication methods (e.g. unidirectional flow) Relies only on capillary forces Possibly reduced enzyme activity upon rehydration (to be tested) Washing and mixing/agitation steps are not possible Cross-reactivity when multiplexing	Increased costs (materials and fabrication equipment) Fabrication expertise (clean room) Increased R&D time Appropriate disposal (depends on the materials e.g. glass)

A reduction of sample volumes (to pL-µL range is possible using microfluidics, as well as simpler handling of complex diagnostic reactions (as valves and chambers in the device do the pipetting work for you). Long-term storage of reagents within the device is also an added value[18]. Microfluidics enable techniques such as "spotting" and multiple surface functionalization that allows multiplexing (i.e., functional integration is possible). Microfluidics furthermore enables a very controlled environment, e.g., in the case of RPA (as we do in chapter 4), a controlled and closed system is required to prevent cross-contamination and amplification of non-specific aerosol DNA which often leads to false positive test results. The microfluidic technology also reduces human-induced error. Microfluidic devices thus have the potential to achieve extreme sensitivity and robustness in resource-limited settings.

Microfluidics for PoC diagnostics is promising, as mass production of thermoplastic polymers allows a cheap cost per unit to enable 'single use and discard' devices. Wax printing is a commonly used method to define flow channels in microfluidic devices. Injection moulding and hot embossing methods are also often used. [16].Fundamental IP rights often have expired, although there may be IP issues for complex or personalized solutions. Although microfluidic devices are more suited to complex assays and reactions, a disadvantage is that the cost per device is unlikely <1$ (i.e., more expensive in comparison to an LFA). Therefore, microfluidics are not suitable for pregnancy or glucose testing which can already be done with cost-effective LFAs. Table 6.1 summarises the pros and cons of lateral flow assays and microfluidic devices. Interestingly, hybrid devices can also be fabricated, which allows developers to combine the advantages of different systems. For example, paper-based microfluidic devices can be fabricated if desired. The Whitesides group has developed many variants of such paper-based microfluidics, which are promising as patterned paper can be used to develop cost-effective bioassays[19,20].

Other detection systems are based on colorimetric, optical/fluorescence, smartphone-based, or electrochemical signalling[21]. In chapter 4 we used DNAzymes (G-quadruplexes) for our readout, as this circumvented the need for labelled oligonucleotide probes, and produced a colorimetric readout that can be seen with the naked eye (label-free detection). Notably, while we used RCA to produce many DNAzymes, the RCA template can also be redesigned to encode a DNA sequence that is complementary to gold nanoparticles which would also produce a visible colorimetric readout upon aggregation of the gold nanoparticles[22]. Optical/fluorescence-based detection systems are very sensitive, but a major limitation is the relatively high costs of the instruments. Smartphones are becoming increasingly popular for imaging and readout, and they can be less subjective than the naked eye. Notably, smartphones are ubiquitous, and app-based data handling (e.g., quantification of LFA lines) have also been developed. For medical diagnostics, a concern is data security (sensitive patient information). Smartphones are also always chang-

ing, and upgrades are always available. Alternatively, smartphones could be used only for computing the data, where a bluetooth connection can be used as opposed to the internet or hardware coupling (susceptible to damage, loss, and add expenses)[23].

In choosing the optimal packaging format for a PoC test, it is important to determine the following:

1. Who is the user?
2. How fast are the results needed?
3. What is the limit of detection of the analyte? This is a very important factor to consider when determining a test format, as this determines the sample volume and possibly the design of the test.
4. What is the expected number of samples?
5. What kind of diagnostic information is needed? Merely a qualitative 'yes/no', or a quantitative estimate of the concentration.
6. What degree of complexity can be handled?
7. Where and how will the data be stored or shared?
8. Which are the relevant strains to develop a test for?
9. What makes clinical sense? Do I need to know the infectious strain to administer treatment?

The answers to these questions will have a major effect on the type of assay that will be developed. The ideal PoC test should meet the ASSURED criteria. In reality, however, there are often performance trade-offs. The ASSURED criteria dictates that the test should be "easy to use". In my opinion, this is rather vague. The ASSURED criteria also dictates an equipment-free test. In my opinion, it is necessary to define 'equipment' here. Although PoC testing aims to reduce human-induced error with fully automated systems, and to streamline and standardise diagnostic procedures, it is important not to overlook the human capacity, or to discredit a laboratory technicians' livelihood by replacing them. Capacity building in resource-limited settings, where people are trained and skills are brought into communities, is important to continue to uplift the community. Indeed, it is important to determine who the end-user is. For example, if the end-user is an employee from Médecins Sans Frontières (MSF), total automation is ideal. If the end-user is an allocated laboratory technician who is trained to perform diagnostic tests, total automation is not required, and is possibly undesirable by the local laboratory technicians. One of the greatest challenges in this field is interpreting the information into a medically

relevant statement, and enabling non-expert users (such as healthcare workers and volunteers) to correctly refer patients.

Summing up, I strongly recommend that our DNA-detection scheme should be incorporated into a microfluidic closed system ("lab-on-a-chip" format), in order for it to be applied as a rapid diagnostic test. The main reason is that the closed microfluidic system will facilitate the sequential reactions that are needed in our DNA-detection scheme (RPA, dCas9 detection and RCA), as well as the extra wash steps that are required between each reaction.

Conditions for the long-term storage of biomolecules and reagents is an important topic. Main parameters to consider and optimize include the storage temperature, the concentration at which the biomolecules are stored, storage time, volume and pH of reagents and buffers, the addition of cryoprotectants, and lyophilization (freeze-drying) agents – all of which have a major effect on the storage stability and activity upon rehydration[24]. Companies have for example investigated whether Cas nucleases can be pre-complexed with the guide RNA and then stored for extended periods of time. Under specific conditions, CRISPR reagents have been shown to be stable for extended periods of time (up to 1 year)[24,24]. In general, CRISPR-based assays are amendable to lyophilization, thus mitigating the need for cold chain management,[1] which is an important advantage.

Nontechnical barriers may hamper the effective implantation of PoC diagnostics. In many endemic regions, diseases such as malaria are often diagnosed presumptively. i.e., febrile patients are presumed to have malaria. This practice developed due to a lack of microscopes and trained microscopists to diagnose febrile patients[25]. During our field research, we learnt that, when available, antimalarial medication is often used before the medication expires (as not using medication before it expires is considered a wasteful practice, thus antimalarial medication is often prescribed to all febrile patients), and the adverse effects are generally negligible. Even after the introduction and widespread availability of malaria rapid diagnostic tests (RDTs), which makes PoC diagnostics of malaria in resource-limited settings possible, febrile patients often receive antimalarials at the community level without a formal diagnosis, due to convenience and lack of knowledge about other febrile-causing diseases[25]. A PoC test that detects both VL and malaria could be an excellent diagnostic tool to test for malaria in endemic regions, as well as maintain surveillance for VL in the early stage of fever presentation. Such multiplexed tests could serve multiple purposes, such as triaging and patient management (prioritize treatment for rapidly developing infections such as malaria, over VL which is a slower progressing disease), rational use of medication to avoid the unnecessary use of antimalarial treatment and antibiotics that contribute to drug resistance, and disease surveillance[26]. Indeed, there is an undoubtable urgent need for new PoC diagnostics that meet

multiple diagnostic needs simultaneously to provide evidence-based treatment[26].

During the review process (chapter 2), we realized that field validation also presents a major final barrier to the implementation of PoC diagnostic tests. During this PhD, we established many valuable collaborations with experts in the field. Upon further development, it is intuitive to use the collaborations that we have developed during this work, such as with MSF, for field validation of our DNA detection scheme. It may also be possible to collaborate with the Drugs for Neglected Diseases initiative (DNDi) to validate the confirmatory (test-of-cure) functionality of our assay by testing the efficacy of VL treatment (although, it is important to note that there are still many open questions about how long parasitic DNA persists in the body after VL treatment). These expert groups would be more suited to address these open questions when developing our DNA-detection scheme as a confirmatory test.

6.3 Final reflections and lessons learned

Developing a new PoC test faces challenges. It is very difficult to move people away from what they know and trust. One solution to this problem is to have them be part of the process to accelerate the uptake of the new technology. Many new technologies have to compete with existing well-known solutions (culture, microscopy). We took the first steps by introducing ourselves to the field early during our research and development, with the intent to make the technology look as familiar as possible to the end user. We have found that developing PoC diagnostics requires a huge investment of time, expertise, and research funding.

This thesis highlighted a few of the key challenges in PoC diagnostics for resource-limited settings, and provided some solutions to overcome them. Despite the global advances and new developments in PoC diagnostics (chapter 1), numerous challenges remain. Sample preparation and field validation are two major challenges for DNA-detection platforms. During the literature research study (chapter 2), we found that for numerous NTDs, sample preparation and standardized diagnostic procedures are limited. In chapter 3, we used an iterative design process to formulate a concept target product profile, which is a useful new tool to match novel technologies to local contexts. In chapter 4, we developed a chitosan-based DNA extraction method that can be applied to both blood and urine samples and explored a diagnostic chain from sample to readout. Promising areas of research that were addressed in this thesis include field-deployable sample preparation, and coupling isothermal amplification to existing detection platforms. Lastly, we explored the inhibition of the Cas9 endonuclease from *Streptococcus pyogenes* (SpCas9) as it may have implications for genome engineering using spCas9.

Key lessons that I learned from this PhD thesis project include:

1. The development time from an idea to a working proof-of-principle is long, longer than the period of one PhD-thesis project.
2. We can lower entry barriers by building on existing technology. One should benefit from existing technologies. For example, the RPA that we employed worked extremely well.
3. A multidisciplinary team is crucial, from scientists to industrial designers to local experts in the field. Our collaboration with industrial design engineers was key as several design iterations are required for a final device and user-instruction manual.
4. An iterative design process is key to matching a novel technology to a local healthcare context.
5. There is a major gap between academic and industrial needs, and thus there is much to learn on regulatory requirements and IP.
6. Merging my scientific interests with social responsibility is truly rewarding on a personal level.

Improved diagnostics reduces misdiagnoses, wasteful treatment practices, and the holistic cost of disease management. The diagnostic test itself may involve cost, but its use will reduce treatment costs. More importantly, it will ensure patient comfort and improve patient outcomes – which, in my opinion, is the ultimate goal of this biomedical research.

References

1. Chiu, C. In Translation Cutting-Edge Infectious Disease Diagnostics with CRISPR. 2015–2017 (2018).
2. Wee, E. J. H., Lau, H. Y., Botella, J. R. & Trau, M. Re-purposing bridging flocculation for on-site, rapid, qualitative DNA detection in resource-poor settings. Chem. Commun. 51, 5828–5831 (2015).
3. Dittrich, S. et al. Target Product Profile for a Diagnostic Assay to Differentiate between Bacterial and Non-Bacterial Infections and Reduce Antimicrobial Overuse in Resource-Limited Settings: An Expert Consensus. PLoS One 11, e0161721 (2016).
4. Adams, E. R. et al. Leishmaniasis Direct Agglutination Test: Using Pictorials as Training Materials to Reduce Inter-Reader Variability and Improve Accuracy. PLoS Negl. Trop. Dis. 6, e1946 (2012).
5. Adams, E. R. et al. Development and Evaluation of a Novel Loop-Mediated Isothermal Amplification Assay for Diagnosis of Cutaneous and Visceral Leishmaniasis. J. Clin. Microbiol. 56, e00386-18 (2018).
6. Nishimasu, H. et al. Crystal Structure of Cas9 in Complex with Guide RNA and Target DNA. Cell 156, 935–949 (2014).
7. Zuo, Z. & Liu, J. Allosteric regulation of CRISPR-Cas9 for DNA-targeting and cleavage. Curr. Opin. Struct. Biol. 62, 166–174 (2020).
8. Globyte, V., Lee, S. H., Bae, T., Kim, J. & Joo, C. CRISPR /Cas9 searches for a protospacer adjacent motif by lateral diffusion . EMBO J. 38, 1–12 (2019).
9. Gootenberg, J. S. et al. Nucleic acid detection with CRISPR-Cas13a / C2c2. 9321, (2017).
10. Gootenberg, J. S. et al. Multiplexed and portable nucleic acid detection platform with Cas13, Cas12a and Csm6. Science (80-.). 360, 439–444 (2018).
11. Chen, J. S. et al. CRISPR-Cas12a target binding unleashes indiscriminate single-stranded DNase activity. Science (80-.). 360, 436–439 (2018).
12. Tsou, J.-H., Leng, Q. & Jiang, F. A CRISPR Test for Detection of Circulating Nuclei Acids. Transl. Oncol. 12, 1566–1573 (2019).
13. Whitesides, G. M. Cool, or simple and cheap? Why not both? Lab Chip 13, 11–13 (2013).
14. Sajid, M. & Daud, M. Designs , formats and applications of lateral flow assay : A literature review. J. Saudi Chem. Soc. 19, 689–705 (2015).
15. Mohd Hanafiah, K. et al. Development of Multiplexed Infectious Disease Lateral Flow Assays: Challenges and Opportunities. Diagnostics (Basel, Switzerland) 7, 51 (2017).
16. Primiceri, E. et al. Key Enabling Technologies for Point-of-Care Diagnostics. Sensors (Basel). 18, 3607 (2018).
17. Sharma, S., Zapatero-Rodríguez, J., Estrela, P. & O'Kennedy, R. Point-of-Care diagnostics in low resource settings: Present status and future role of microfluidics. Biosensors 5, 577–601 (2015).
18. Sackmann, E. K., Fulton, A. L. & Beebe, D. J. The present and future role of microfluidics in biomedical research. Nature 507, 181–189 (2014).
19. Martinez, A. W., Phillips, S. T. & Whitesides, G. M. Diagnostics for the Developing World : Microfluidic Paper-Based Analytical Devices. 82, 3–10 (2010).
20. Akram, M. S., Lowe, C. R., Yetisen, A. K., Akram, M. S. & Lowe, C. R. Paper-based microfluidic point-of-care diagnostic devices . Lab Chip. (2016). doi:10.1039/c3lc50169h
21. Pashchenko, O., Shelby, T., Banerjee, T. & Santra, S. A Comparison of Optical, Electro-

chemical, Magnetic, and Colorimetric Point-of-Care Biosensors for Infectious Disease Diagnosis. ACS Infect. Dis. 4, 1162–1178 (2018).
22. Hu, B. et al. A sensitive colorimetric assay system for nucleic acid detection based on isothermal signal amplification technology. Anal. Bioanal. Chem. 409, 4819–4825 (2017).
23. Majumder, S. & Deen, M. J. Smartphone Sensors for Health Monitoring and Diagnosis. Sensors (Basel). 19, 2164 (2019).
24. Alt-R CRISPR reagent stability in storage and with freeze-thaws | IDT. Available at: https://eu.idtdna.com/pages/education/decoded/article/stability-of-crispr-reagents-under-different-freezing-storage-and-thawing-conditions
25. Harvey, S. A. Observe Before You Leap: Why Observation Provides Critical Insights for Formative Research and Intervention Design That You'll Never Get From Focus Groups, Interviews, or KAP Surveys. Glob. Heal. Sci. Pract. 6, 299–316 (2018).
26. Hotez, P. J., Pecoul, B., Rijal, S., Boehme, C. & Aksoy, S. Eliminating the Neglected Tropical Diseases : Translational Science and New Technologies. 1–14 (2016). doi:10.1371/journal.pntd.0003895.
27. Koczula, K. M. & Gallotta, A. Lateral flow assays. Essays Biochem. 60, 111–120 (2016).
28. Posthuma-Trumpie, G. A., Korf, J. & van Amerongen, A. Lateral flow (immuno)assay: its strengths, weaknesses, opportunities and threats. A literature survey. Anal. Bioanal. Chem. 393, 569–582 (2009).

Summary

As is clear in this time of a global pandemic, rapid and reliable diagnosis of infectious diseases is essential. While COVID-19 holds the world in its grip, more than 1 billion people suffer from Neglected Tropical Diseases (NTDs). Effective diagnostics are essential for disease management and to guide treatment strategies during the surge of infectious outbreaks, disease surveillance and possible disease eradication. While treatment is often available, NTDs are endemic among marginalized populations due to the unavailability or inadequacy of point-of-care (PoC) diagnostic tests which cause empirical misdiagnoses. The work presented in this thesis first sketches the context of use and barriers to diagnosing infectious diseases in resource-limited settings, and then focusses on applying this knowledge to develop a DNA-based PoC diagnostic test for use within such settings.

In order to identify the needs, we reviewed the status quo of diagnostic tests and practices for all 24 NTDs, as defined in the WHO's 2021-2030 roadmap based on their different diagnostic requirements, see chapter 2. We found that there is a clear need for improved and/or new PoC diagnostic tests. We identified diagnostic needs in the context of NTDs, and outlined technical requirements and guidelines for the development of novel PoC tests that would fit endemic resource-limited settings. Throughout the review process, it became increasingly evident that there is a lack of field-validated PoC diagnostic tests for NTDs. Furthermore, we realized that a technical success does not automatically translate into a commercial success. More specifically, there are many more published proof-of-principle studies than there are field validated tests. The disconnection between the laboratory and the field was found to be rooted in technical issues such as field-deployable sample preparation, but also in lack of awareness and unavailability of PoC tests. We highlighted the importance of awareness programs and availability of PoC tests where they are needed the most.

To gain an enhanced understanding of the context of use, we conducted direct observations in the field during a two-week field study in the remote regions of Kenya and Uganda. In chapter 3, we presented the results of this field research wherein we explored the local healthcare systems in these rural resource-limited settings. The availability of resources (i.e., infrastructure, electricity, skills) and subsequent design specifications for a DNA-based PoC test are described in this chapter. We presented our stepwise approach which integrates elements of design thinking which facilitated the identification (for ourselves as researchers) and communication (to other stakeholders) of barriers to diagnosing visceral leishmaniasis (VL) in these two East African countries. Our approach uses a combination of literature reviews and field research that presents a context analysis of the healthcare

systems and diagnostic practices in these two countries. In this chapter, we used two examples of visual thinking, Gigamaps and patient journeys, to communicate our findings from the field with key stakeholders such as Médecins Sans Frontières (MSF) and local public healthcare officers. As a result, we formulated a concept target product profile (CTPP), which is a new design tool that will enable researchers to match research and development of novel PoC diagnostic tests to the local healthcare context in which the test will be used. We formulated a CTPP for two diagnostic use cases, namely screening & confirmation, and test-of-cure. These use cases were identified by healthcare specialists as a priority for novel PoC diagnostic tests for visceral leishmaniasis.

Versatile and rapid portable sensing of nucleic acids will improve applications in healthcare. In chapter 4, we presented the scientific methodology that we used to develop a novel isothermal DNA-based detection scheme that can be applied in resource-limited settings for diagnostics. The key scientific innovation in this chapter is to couple the CRISPR-dCas9 system with a colorimetric read-out that is visible to the naked eye. Using the context-specific knowledge that we gained in chapters 2 and 3, we developed an isothermal DNA-detection scheme that has the potential to be used as a diagnostic test for NTDs. Using a pH based chitosan-mediated approach, DNA was extracted and amplified using recombinase polymerase amplification with a sensitivity of <10 copies of DNA in a sample in a temperature range of 25ºC to 45ºC within 15 minutes. Target DNA was bound by dCas9 that was labelled with a DNA sequence to induce rolling circle amplification (RCA), which can be conducted from 15ºC to 60ºC. RCA produced many copies of a G quadruplex DNA structure which facilitate a colorimetric readout that is visible to the naked eye. To visualise a colour change, the RCA reaction required 24 hours at room temperature, coupled to 15 minutes incubation time for the colour development. Finally, we provide an example where we employ this DNA detection scheme to diagnose visceral leishmaniasis (VL), which is a fatal disease for which there is treatment available, that urgently requires improved PoC diagnostic tests in Sub-Saharan Africa. We envision that this diagnostic scheme can be applicable for the detection of any parasitic DNA in bodily fluids and will be broadly applicable worldwide.

The key scientific innovation in this thesis is the use of the CRISPR-dCas9 system as a DNA-detection tool. Although this thesis mainly focused on the development of a context-specific DNA-based PoC test, we explored the inhibition of SpCas9 by small RNA molecules as it may have implications for genome engineering using Cas9. This thesis thus presents both applied and fundamental components of Cas9. Various factors have been found to affect Cas9 cleavage activity. Mismatches between the guide RNA (gRNA) and target DNA can decrease cleavage and secondary structures of target DNA such as G-quadruplexes can also greatly decrease cleavage by Cas9. In chapter 5, we presented a study that demonstrated that Cas9 activity can be inhib-

ited by small RNA molecules in vitro. Biochemical assays revealed that sequences which are complementary to the seed region of the single guide RNA (sgRNA) inhibit cleavage progressively with increasing ratios of inhibitor to sgRNA. Single-molecule fluorescence assays revealed that the hybrids (duplexes) are efficiently loaded into the Cas9 protein and prevent further interactions of the protein with the DNA target. Finally, we demonstrated that a trigger sequence, that is complementary to the inhibitor sequence, can remove the inhibitor sequence from the Cas9 ribonucleoprotein (RNP) complex if it is hybridized to the end of the sgRNA (PAM distal), but not the seed region. Upon release of the inhibitor from the Cas9 RNP complex, we speculated that Cas9 remains stuck in an inactive conformation and cannot resume binding activity to the DNA target. Such inhibition may have implications for genome engineering strategies, where miRNA can potentially base-pair with the gRNA and inhibit cleavage activity, or transcriptionally active genes can be targeted less efficiently. In vivo experiments need to be conducted to explore the extent of these inhibition observations.

Finally, in chapter 6, I presented a perspective on the adaptable potential of the DNA-detection scheme that we developed in chapter 4. Determining when and where a PoC test will be used puts constraints on the test format. Upon presenting an overview of lateral flow assays and microfluidic systems, I strongly recommend that our DNA-detection scheme should be incorporated into a closed microfluidic system in order for it to be applied as a point-of-care diagnostic test, as that allows the multiple sequential reactions and wash steps that are needed in our DNA-detection scheme. Finally, I provided a few concluding thoughts on the barriers to diagnosing infectious diseases such as VL in resource-limited settings, and I shared some lessons learned. Overall, it is my hope that the scientific work reported in the thesis will eventually contribute to improving the health of the many people that are suffering from NTDs.

Samenvatting

Zoals duidelijk te zien is in deze tijd met een wereldwijde pandemie, is een snelle en betrouwbare diagnose van infectieziekten essentieel. Terwijl COVID-19 de wereld in zijn greep houdt, lijden er meer dan 1 miljard mensen aan Verwaarloosde Tropische Ziektes, ook wel Neglected Tropical Diseases (NTDs) genoemd. Effectieve diagnostiek is essentieel voor ziektebeheersing en voor de sturing van behandelingsstrategieën tijdens infectie-uitbraken, ziektesurveillance en mogelijke uitroeiing van deze ziektes. Hoewel een behandeling in veel gevallen beschikbaar is, zijn NTDs endemisch onder gemarginaliseerde bevolkingsgroepen wegens niet-beschikbare of ontoereikende point-of-care (PoC) diagnostische tests, met empirisch verkeerde diagnoses als gevolg. Het werk dat in dit proefschrift wordt gepresenteerd, schetst allereerst de gebruikscontext en de barrières voor het diagnosticeren van infectieziekten in een omgeving met beperkte middelen, en richt zich vervolgens op het toepassen van deze kennis voor de ontwikkeling van een op DNA gebaseerde PoC-diagnostische test, voor gebruik binnen een dergelijke omgeving.

Om de behoeften te identificeren, hebben we een overzicht gemaakt van de status quo betreffende diagnostische tests en de uitvoering ervan met betrekking tot alle 24 NTDs, zoals gedefinieerd in het 2021-2030 beleidsplan van de WHO (hoofdstuk 2). Het blijkt dat er een duidelijke behoefte is aan verbeterde en/of nieuwe PoC-diagnostische tests. We identificeerden diagnostische behoeften in de context van NTD's en schetsten technische vereisten en richtlijnen voor de ontwikkeling van een nieuwe PoC test die past binnen endemische gebieden met beperkte middelen. Gedurende dit proces, werd het steeds duidelijker dat er een gebrek is aan in het veld gevalideerde PoC diagnostische tests voor NTD's. Bovendien realiseerden we ons dat een technisch succes zich niet automatisch vertaalt in een commercieel succes. Meer specifiek zijn er veel meer gepubliceerde conceptonderzoeken dan veld-gevalideerde tests. De kloof tussen het laboratorium en het veld blijkt te zijn geworteld in technische problemen zoals in-het-veld inzetbare monstervoorbereiding, maar ook in een gebrek aan bewustzijn en niet-beschikbare PoC-tests. We benadrukten het belang van bewustwordingsprogramma's en van de beschikbaarheid van PoC-tests op de plekken waar ze het meest nodig zijn.

Om de gebruikerscontext beter te begrijpen, hebben we directe observaties in het veld gedaan tijdens een veldstudie van twee weken in de rurale regio's van Kenia en Oeganda. In hoofdstuk 3 presenteerden we de resultaten van dit veldonderzoek waarin we de lokale gezondheidszorgsystemen in deze rurale gebieden met beperkte middelen verkenden. De beschikbaarheid van bepaalde middelen (infra-

structuur, elektriciteit, vaardigheden) en de daaruitvolgende ontwerpspecificaties voor een op DNA gebaseerde PoC-test worden in dit hoofdstuk beschreven. We presenteerden onze stapsgewijze aanpak met geïntegreerde elementen van ontwerpgestuurd denken, die de identificatie (voor onszelf als onderzoekers) en communicatie (naar andere belanghebbenden) van de barrières voor de diagnostiek van viscerale leishmaniasis (VL) in deze twee Oost-Afrikaanse landen faciliteerde. Onze aanpak maakte gebruik van een combinatie van literatuuronderzoek en veldonderzoek, waarmee we een contextanalyse hebben gepresenteerd van de gezondheidszorgsystemen en diagnostische praktijken in deze twee landen. In dit hoofdstuk hebben we twee voorbeelden van visueel denken gebruikt, Gigakaarten en patiëntreizen, om onze bevindingen uit het veld te communiceren met de belangrijke belanghebbenden zoals Artsen Zonder Grenzen (AZG) en lokale gezondheidsfunctionarissen. Als resultaat hebben we een concept doelproductsprofiel (concept target product profile, CTPP) geformuleerd, een nieuwe ontwerpmethode waarmee onderzoekers onderzoek en ontwikkeling van nieuwe PoC-diagnostische tests kunnen afstemmen op de lokale gezondheidszorgcontext waarin de test zal worden gebruikt. We hebben een CTPP opgesteld voor twee diagnostische gebruiksgevallen, namelijk screening & bevestiging en testen op genezing. Deze gebruiksgevallen werden door specialisten in de gezondheidszorg geïdentificeerd als een prioriteit voor nieuwe PoC-diagnostische tests voor viscerale leishmaniasis.

Veelzijdige, snelle en draagbare detectie van nucleïnezuren zal de toepassingen in de gezondheidszorg verbeteren. In hoofdstuk 4 hebben we de wetenschappelijke methodologie gepresenteerd die we hebben gebruikt om een nieuw isothermisch DNA-gebaseerd detectieschema te ontwikkelen dat kan worden toegepast voor diagnostiek in een omgeving met beperkte middelen. De belangrijkste wetenschappelijke innovatie in dit hoofdstuk is het koppelen van het CRISPR-dCas9 systeem aan een kleuruitlezing die met het blote oog zichtbaar is. Met behulp van de context-specifieke kennis die we in hoofdstuk 2 en 3 hebben opgedaan, hebben we een isothermisch DNA-detectieschema ontwikkeld dat potentieel gebruikt kan worden als diagnostische test voor NTD's. Met behulp van een op pH gebaseerde chitosan-gemedieerde benadering werd DNA geëxtraheerd en geamplificeerd met behulp van recombinase-polymerase-amplificatie met een gevoeligheid van <10 kopieën van DNA in een monster, in een temperatuurbereik van 25ºC tot 45ºC binnen 15 minuten. Doel-DNA werd gebonden door dCas9 dat werd gelabeld met een DNA sequentie om rollende cirkel amplificatie (RCA) te induceren, welke kan worden uitgevoerd bij een temperatuur van 15ºC tot 60ºC. RCA produceerde veel kopieën van een G-quadruplex DNA-structuur wat een kleuruitlezing mogelijk maakt die zichtbaar is voor het blote oog. De RCA-reactie had 24 uur nodig om een kleurverandering te visualiseren bij kamertemperatuur, met 15 minuten incubatietijd voor de kleurontwikkeling. Tot slot geven we een voorbeeld waarin we dit DNA-detectieschema gebruiken om viscerale leishmaniasis (VL) te diagnosticeren.

VL is een behandelbare dodelijke ziekte die dringend verbeterde PoC-diagnostische tests vereist in Sub-Sahara Afrika. We voorzien dat dit diagnostische schema van toepassing kan zijn op de detectie van elk parasitair DNA in lichaamsvloeistoffen en wereldwijd breed toepasbaar zal zijn.

De belangrijkste wetenschappelijke innovatie in dit proefschrift is het gebruik van het CRISPR-dCas9 systeem als een instrument voor DNA-detectie. Hoewel dit proefschrift zich voornamelijk richtte op de ontwikkeling van een contextspecifieke DNA-gebaseerde PoC test, hebben we ook de remming van SpCas9 door kleine RNA-moleculen onderzocht, wat implicaties kan hebben voor genetische manipulatie met Cas9. Dit proefschrift presenteert dus zowel toegepaste als fundamentele componenten van Cas9. Er zijn verschillende factoren gevonden die de splitsingsactiviteit van Cas9 beïnvloeden. Mismatches tussen het enkele gids-RNA (single guide RNA of sgRNA) en doel-DNA kunnen de splitsing verminderen, en ook secundaire structuren van het doel-DNA zoals G-quadruplexen kunnen de splitsing door Cas9 sterk verminderen. In hoofdstuk 5 presenteerden we een studie die aantoonde dat Cas9-activiteit in vitro kan worden geremd door kleine RNA-moleculen. Biochemische testen toonden aan dat sequenties, die complementair zijn aan het kiemgebied van het sgRNA, de splitsing progressief remden met een toenemende verhouding van remmers tot sgRNA. Enkel-molecuul fluorescentie experimenten toonden aan dat de hybride duplexen efficiënt in het Cas9-eiwit worden geladen en verdere interacties van het eiwit met het doel-DNA voorkomen. Tenslotte hebben we aangetoond dat een trekkersequentie, die complementair is aan de remmersequentie, de remmersequentie uit het Cas9 ribonucleoproteïne (RNP) complex kan verwijderen als het wordt gehybridiseerd aan het uiteinde van het sgRNA (PAM distaal), maar niet aan het zaadgebied. Na het vrijkomen van de remmer uit het Cas9 RNP complex, speculeerden we dat Cas9 vast blijft zitten in een inactieve conformatie en de bindingsactiviteit aan het DNA-doelwit niet kan hervatten. Een dergelijke remming kan implicaties hebben voor genetische manipulatiestrategieën, waardoor micro-RNA misschien kan hybridiseren met het gids-RNA en zo splitsingsactiviteit kan remmen of actieve genen minder efficiënt gemanipuleerd kunnen worden. In vivo experimenten moeten nog worden uitgevoerd om de omvang van deze waarnemingen te onderzoeken.

Tot slot presenteerde ik in hoofdstuk 6 een perspectief op de aanpasbaarheid van het DNA-detectieschema dat we in hoofdstuk 4 hebben ontwikkeld. Het bepalen wanneer en waar een PoC-test zal worden gebruikt, legt beperkingen op aan de testprocedure. Bij het presenteren van een overzicht van laterale stromingstesten en microfluïdische systemen, raad ik ten zeerste aan om ons DNA-detectieschema op te nemen in een gesloten microfluïdisch systeem zodat het kan worden toegepast als een snelle diagnostische test, aangezien deze opzet de meerdere opeenvolgende reacties en was-stappen mogelijk maakt die nodig zijn in ons DNA-detec-

tieschema. Ik beëindig dir proefschrift met enkele opmerkingen over de barrières voor het diagnosticeren van infectieziekten zoals VL in omgevingen met beperkte middelen.

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I can do all things through Christ who strengthens me
Philippians 4 verse 3

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Team Photo at Kenya Medical Research Institute (KEMRI), Nairobi, Kenya. November 2018. Left to Right: Dr. Johnstone Ingonga, Ms. Hellen Nyakundi, Astrid ten Bosch, Michel Bengtson, Dr. Damaris Matoke-Muhia, Prof. Cees Dekker, and Dr. Mitasha Bharadwaj.

I would like to thank my supervisor Cees Dekker for the opportunity to work in your research group. What a journey! Despite our shared concerns at the start, we embarked on this journey, and I appreciate this experience more than words

can convey. Cees, you have created a work atmosphere where critical thinking is fostered in each of us. My most fond memory of you is singing praise and worship together in rural Kenya. Despite not having very many worldly possessions, the locals sang a song with us that loosely translates to “Thank you God for giving us everything that we need”. This was a very special moment of reflection for me, and I will forever appreciate this experience. I admire your commitment to our faith and to science. It is truly remarkable to see the enthusiasm that you have, and the dedication to balancing it all.

Kenya. November 2018. Prof. Cees Dekker, Michel Bengtson, and locals.

Kenya. November 2018. Prof. Cees Dekker and Michel Bengtson.

BN is such a dynamic department. To the people at BN, thank you for sharing your journeys with me. A special thank you to Dr. Chirlmin Joo and Dr. Stan Brouns for sharing their groups expertise with me throughout the years. Amanda, things would not run as smoothly without you, thank you for your support. A very special thank you to Dijana as well for all your support when I joined BN.

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Being in such a large group brings so many personalities into a melting pot, and I admire how well you all work together. I have often thought about the amount of time that the CDlab members spend together outside of work, whether it is climbing, playing squash, forming multiple bands, mid-week drinks, dinners – the fact that we all choose to spend our spare time together is testament to the wonderful people that make up this group. Jaco, by far the best person to work with in a wet lab, thank you for all your support throughout this PhD. You are a brilliant molecular scientist, and a really great friend. I have always considered you to be my mentor. I have thoroughly enjoyed our conversations in the lab, everything from running gels to raising a child in the Netherlands and African safaris. The diagnostics team: Thank you Felix and Greg for supporting me during the initial phase of this project. Parul, thank you for the time that we spent together. My postdocs, Mitasha and Oskar, thank you for believing in this project as much as I do. I appreciate the time and effort spent on developing the project together. You are both amazing scientists, and I appreciate everything that you have taught me along the way. Mitasha and Shobit, congratulations on the birth of Nayomi. To the technical support in the CDlab, it would not be possible for any of us to conduct our research without you. Allard, thank you from the bottom of my heart for helping me get my apartment ready for Madison (AFM by day, and assembling cribs by night). Eli, Ashmiani, Esengül, I thoroughly enjoyed our time together, thank you for always taking a moment out of your busy days to check in. Theo, I have lost count of how many gels I “borrowed” from you, thank you for always supporting me in the lab. Jacob, by far the most knowledgeable yet humble person that I have ever met, I enjoyed speaking to you about life. My most fond memory is our plane trip to Ireland. I was

filled with anxiety after leaving Madison for the first time, and unbeknown to you (and Allard), the deeply honest conversation that we had on the plane really put me at ease. Thank you also for this beautiful cover, I enjoyed the process of creating it and appreciate your time spent on it.

I had the honour of supervising (and co-supervising) amazing students during my PhD. To Nicky, Valerie, Wiebeke, Paula, Menno, Ramon, Veronique, Loulotte, and Lucas, thank you all for contributing to our diagnostics research. I had so much fun working with you all. Bas, it was great sharing an office with you. Astrid, a special thank you for your contribution to my PhD, I am extremely proud of the field work that we all did together, and so happy to have gotten to know you more thereafter.

To the JooCs, Viktorija and Thijs. Thank you for sharing your expertise with me. Our collaboration was such a wonderful experience for me, and I am beyond grateful for the friendships that have blossomed.

JC, where do I begin? You have played an integral role in my PhD. A core chapter in my thesis reflects the design nature of your work, and I cannot thank you enough for this contribution to my thesis and for being such a great mentor to me as well. Your energy is what is really infectious! I admire your passion for global development. Not surprisingly, all the DGI fellows gravitate towards you. Pre-COVID19, I often worked in IDE as the atmosphere is so welcoming. You would bump into me at the IDE coffee corner quite often and ask if we had a meeting that you forgot about. I truly enjoyed working with you, and am elated that we get to continue working together on the INSPiRED project. Lisette, it has been an absolute pleasure working in your group, thank you for the constructive feedback on my thesis, and I look forward to contributing to the fight against NTDs. To my brother Tope, I am so happy that we finally get to work together on a project that is close to my heart. A very special thank you to you and Irene for welcoming us into your family.

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An African proverb says 'It takes a village to raise a child'. Indeed! Hence, to my village, thank you, you have played a vital role in our lives. Madison has the best honorary aunts and uncles, who just so happen to be some of the best budding scientists that I know. To (uncles) Wayne, Tony, Berto, and Oskar, thank you for being amazing male role models. I enjoy the juxtaposing memories when I look back at Sinterklaas photos of you all playing with LEGO trains and eating playdough ice-creams, whilst stressing about hardcore research. Wayne, on the day that you got off the bike to walk with me, I could not have imagined how close we would become (a sentiment that I am sure you share with me). You are a great scientist, a truly great friend, and (surprisingly) an even better chef. Birthday steaks are one of the highlights of my year. To the best tax man that a girl could ask for, thank you Tony for your never-ending willingness to help and more importantly, the never-ending supply of apple pie. Berto, thank you for stepping in to spend time with Madison when we needed. I can hardly believe how blessed we are to have met you. Oski, you are among the kindest souls I have ever met. You gave me a boost of much-needed positivity and whether it was G&T night or yet another colorimetric reaction, I was always laughing. To (aunties) Laura, Ana, Mireia, Gro, Paola and Sabina, thank you for all the time that you have spent with us. You ladies have supported me in more ways than I can express in words. Gro, I am so excited for your journey into motherhood, congratulations! Mireia, I will cherish all your wonderful recipes! Paola, thank you for all your support during the pandemic, I appreciate our friendship and am so happy for this next big step in your life! Sabina, although I do not see you as often as I used to, when I do see you, it feels so natural, as though I see you every day. Thank you for including Madison and me in all your special occasions. Ana, thank you for taking care of Madison so that I could pursue field research in East Africa. I will forever be grateful for this ultimate act of trust and friendship. Viktorija, Seb and Matilda; Zhanar and Allen; you have all made our lives rich with love and laughter. Laura and Taylor; Sandra and John, thank you for opening your homes and hearts to Madison and me. I appreciate coming home to such wonderful neighbours. It warms my heart to see Madison run in and out so happily to see you all. Monique, Richard, Sterre, Zara, Fay, Hennie and extended family, thank you making Madison and I part of your family. Moving to the Netherlands was all the more special because of you all. To Yro, Theo, Isaac (and parents); thank you for all the playdates and celebrating all the special occasions with Madison and me.

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much. We have been blessed beyond measure. To my family, aunty Nola and uncle Clive; Jody and Dan; aunty Cheryl and uncle Kiki, Tammy, Tash, Colux and Cherise, thank you for your support that spans oceans! Jodes, thank you for all the trips to Delft, it meant the world to me. To my best friend Amber, it is rare to find friendships that span decades. We have grown up together, and now share the experience of raising our daughters. Georgia and Alaia are blessed to have you as their mom. Thank you for your endless support, and for bringing so much joy and laughter into my life.

Finally, a word for Madi. They say that 'children are Gods expression of hope', and I concur. Madi, you are my greatest blessing of all. You give me renewed purpose, meaning, and direction. You have given me the balance in my life that I truly value, and I cannot wait to see what the future holds for us. I love you with all my heart.

Curriculum Vitae

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List of publications

5. Globyte V*., **Bengtson M***., van der Torre J., Dekker C., Joo C. Small RNA molecules inhibit the catalytic activity of Cas9 in vitro. Manuscript in preparation.

4. **Bengtson M***., Bharadwaj M*., Franch O*., van der Torre J., Meerdink V., Schallig H., Dekker C. CRISPR-dCas9 based DNA detection scheme for diagnostics in resource-limited settings. Manuscript in preparation.

3. Bharadwaj M*., **Bengtson M*.**, Golverdingen M., Wailing L., Dekker C. Diagnosing point-of-care diagnostics for neglected tropical diseases. Submitted to *PLoS NTDs* October 2020.

2. **Bengtson M***., Bharadwaj M*., ten Bosch A., Nyakundi H., Matoke-Muhia D., Dekker C., and Diehl J.C. Matching Development of Novel Point-of-Care Diagnostic Tests to the Local Context: A Case Study of Visceral Leishmaniasis in Kenya and Uganda. *Glob Health Sci Pract. 2020;8(3).* Accepted August 2020. doi: https://doi.org/10.9745/GHSP-D-20-00028.

1. Yang W., Restrepo-Pérez L**.**, **Bengtson M**., Heerema S.J., Birnie A., van der Torre J., Dekker C. Detection of CRISPR-dCas9 on DNA with Solid-State Nanopores. Nano Lett. 10, 6469-6474 (2018). doi: https://doi.org/10.1021/acs.nanolett.8b02968.

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