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UNIVERSITY OF TARTU
Press

Institute of Technology, Faculty of Science and Technology, University of Tartu, Estonia

This dissertation was accepted for the commencement of the degree of Doctor of Philosophy in biomedical technology on 22nd of November 2017 by the Council of the Institute of Technology, Faculty of Science and Technology, University of Tartu, Estonia.

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Commencement: Auditorium 121, Nooruse 1, Tartu, Estonia, at 13:15 on January 26th, 2018

Publication of this dissertation is granted by the Institute of Technology, Faculty of Science and Technology, University of Tartu

ISSN 2228-0855
ISBN 978-9949-77-639-9 (print)
ISBN 978-9949-77-640-5 (pdf)

University of Tartu Press
www.tyk.ee

ABSTRACT

Sexually transmitted infections (STIs) have become a serious healthcare burden, with more than a million new cases acquired every day worldwide. The majority of STIs are asymptomatic, causing no discomfort to the patient, which further promotes their spread. Screening high-risk groups for STIs has been proven to be an effective strategy to restrict their spread. This requires support of efficient and fast diagnostics. Laboratory-based techniques offer a high level of diagnostic accuracy, but they are often time-consuming and require expensive machinery and well-trained personnel. To date, the lack of cost-efficient, reliable point-of-care (POC) diagnostics has been considered the major barrier for STI control and prevention. Such tests could enable diagnosis and treatment within the same clinical visit.

Chlamydia trachomatis is the most spread STI with 131 million new cases annually. Most POC tests for *C. trachomatis* diagnostics are based on immunoassay, with the major drawback of low sensitivity levels.

The research presented in this thesis primarily addresses the issue of bringing highly accurate and reliable nucleic acid-based diagnostics to POC applications. We have developed a recombinase polymerase isothermal nucleic acid amplification-based assay for specific *C. trachomatis* detection. The amplification takes only 10 min to produce a naked-eye readable result using lateral-flow strip technology for product detection. The assay is highly sensitive and specific with 100% (95% CI, 70%–100%) clinical sensitivity and 100% (95% CI, 92%–100%) specificity.

Besides instrument-free application, one of the major advantages of isothermal amplification is its higher tolerance towards biological sample components. In our case, this enabled *C. trachomatis* target amplification directly from urine samples without prior nucleic acid extraction. The use of heat-treated urine samples for direct amplification resulted in 83% (95% CI, 51%–97%) clinical sensitivity, which is significantly higher than the sensitivity of *C. trachomatis* immunoassays. The possibility of omitting the nucleic acid purification step is a great advantage for POC applications. However, two aspects must be considered when amplifying directly from clinical samples: biological specimen inhibition and sample pretreatment requirements.

PCR is highly sensitive towards biological sample components, with inhibition of the reaction observed even when traces of the specimen remain in the extracted nucleic acid samples. We have found that loop-mediated isothermal amplification (LAMP) can tolerate the addition of up to 20% urine without significant impact on the amplification speed. We have also shown that high Na^+ concentration is responsible for inhibition of urine samples on LAMP and that the inhibitory effect is mainly executed through affecting polymerase activity. Thus selection of more tolerant DNA polymerases is the most effective strategy to overcome clinical sample inhibition of the amplification reaction.

Sample pretreatment prior to downstream amplification is often required to release nucleic acid material from cells. We have compared the efficiency of different sample pretreatment strategies such as heat, alkali, detergent, and lysozyme treatment. We have also introduced a novel sample pretreatment method using membrane-active antimicrobial peptides (AMPs). Our results showed that proper sample pretreatment can significantly increase downstream amplification efficiency, and AMP treatment worked best for *E. coli* detection from urine samples.

In conclusion, this work explores the feasibility of nucleic acid amplification directly from clinical samples, presenting initial clinical efficiency data and addressing clinical sample inhibition and pretreatment issues.

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LIST OF ORIGINAL PUBLICATIONS

The following publications form the basis of the dissertation and are referred to in the text using Roman numerals:

I. **Krõlov, K.**, Frolova, J., Tudoran, O., Suhorutsenko, J., Lehto, T., Sibul, H., Mäger, P., Laanpere, M., Tulp, I., and Langel, Ü. (2014) Sensitive and rapid detection of *Chlamydia trachomatis* by recombinase polymerase amplification directly from urine samples. J.Mol. Diagn. 16(1), 127–135.

II. Jevtuševskaja, J., **Krõlov, K.,** Tulp, I., and Langel, Ü. (2017) The effect of main urine inhibitors on the activity of different DNA polymerases in loop-mediated isothermal amplification. Expert Rev Mol Diagn. 2017 Apr;17(4):403–410.

III. **Krõlov**, K., Uusna, J., Grellier, T., Andresen, L., Jevtuševskaja, J., Tulp, I., and Langel, Ü. (2017) Implementation of antimicrobial peptides for sample preparation prior to nucleic acid amplification in point-of-care settings. Expert Rev Mol Diagn. 2017 Oct 9:1–9.

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Author's contribution to each article is as follows:

I. Designed the experiments and analyzed the data, participated in the experimental work, participated in the writing of the manuscript as a corresponding author;
II. Participated in the experimental design and data analysis, performed some of the experiments, participated in the writing of the manuscript;
III. Designed and performed the experiments, analyzed the data, participated in the writing of the manuscript as a corresponding author;

Other publications:

IV. Jevtuševskaja J, Uusna J, Andresen L, **Krõlov** K, Laanpere M, Grellier T, Tulp I, Langel Ü. (2016) Combination with antimicrobial peptide lyses improves loop-mediated isothermal amplification based method for Chlamydia trachomatis detection directly in urine sample. BMC Infect Dis. 2016 Jul 13;16:329.
V. **Viikov K,** Jasnovidova O, Tamm T, Sedman J. (2012) C-terminal extension of the yeast mitochondrial DNA polymerase determines the balance between synthesis and degradation. PLoS One. 2012; 7(3): e33482.
VI. **Viikov K,** Väljamäe P, Sedman J. Yeast mitochondrial DNA polymerase is a highly processive single-subunit enzyme. Mitochondrion. 2011 Jan;11(1):119–26.

ABBREVIATIONS

AMP	antimicrobial peptide
BSA	bovine serum albumin
CI	confidence interval
DFA	direct immunofluorescence assay
EIA	enzyme immunoassay
FAM	6-carboxyfluorescein
FDA	U.S. Food and Drug Administration
gDNA	genomic DNA
HBV	hepatitis B virus
HDA	helicase-dependent amplification
HIV	human immunodeficiency virus
HPV	human papillomavirus
HSV	herpes simplex virus
LAMP	loop-mediated isothermal amplification
LF	lateral-flow
LGV	lymphogranuloma venereum
LOD	limit of detection
LPS	lipopolysaccharide
MOMP	major outer membrane protein
NAAT	nucleic acid amplification techniques
NASBA	nucleic acid sequence-based amplification
NEAR	nicking enzyme amplification reaction
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PID	pelvic inflammatory disease
POC	point-of-care
RCA	rolling circle amplification
RT	room temperature
RPA	recombinase polymerase amplification
SAS	signal amplification system
SDA	strand displacement amplification
SSB	single-strand DNA binding protein
STI	sexually transmitted infection
TMA	transcription mediated amplification
TRF	time-resolved fluorescence
WHO	World Health Organization

INTRODUCTION

Sexually transmitted infections are a major reproductive health concern worldwide. Several STIs are associated with infertility, mother-to-child transmission, and adverse birth outcomes, in addition to the increased risk of HIV acquisition.

Most prevalent STIs, such as *Chlamydia trachomatis* and *Neisseria gonorrhea,* are effectively curable but require accurate and fast diagnosis, as they are majorly asymptomatic. While accurate diagnostics of the STIs and appropriate screening programs are widely used in high-income countries to address this health issue, low- and middle-income countries often lack access to affordable, time-efficient and geographically available diagnostics facilities. This is leading to low follow-up rates among patients and impeded treatment of the STIs, further promoting their spread.

The availability of affordable rapid tests for infectious disease diagnostics is addressed by point-of-care (POC) diagnostics. In addition to being user-friendly and robust, rapid POC tests are required to be sensitive and specific, to gain an accurate diagnostic result, and preferably to be instrument-free. Application of POC tests for STI diagnostics is a way to restrict their spread and relieve their burden on the healthcare system.

Currently, two STI tests meet these requirements: syphilis and HIV POC tests, which are both based on immunochromatographic antibody detection using lateral-flow technology. Although applicable in some cases, the majority of these tests fail to produce sensitivity and specificity that could meet laboratory level diagnostics. Therefore, alternative methods are being explored for pathogen POC diagnostics.

Nucleic acid amplification-based techniques (NAAT) are highly sensitive and specific methods that have been widely applied in laboratories. However, their application for POC purposes is restricted due to several requirements. The recent emergence of isothermal amplification techniques has enabled instrument-free performance of the NAAT, which is a major step towards POC applications. Still, several obstacles have yet to be overcome, such as sample processing and nucleic acid preparation prior to amplification. Direct amplification from minimally processed or unprocessed clinical samples is one opportunity that is addressed in this thesis.

1. LITERATURE OVERVIEW

1.1. Sexually transmitted infections

There are more than 30 different bacteria, parasites, and viruses that are spread by sexual contact, causing over 1 million infections every day worldwide (WHO). The most prevalent sexually transmitted infections are *Chlamydia trachomatis, Neisseria gonorrhoeae, Treponema pallidum* (syphilis), and *Trichomonas vaginalis*. They are responsible for 357 million cases annually and are currently curable (WHO). However, bacteria resistance to antibiotics has increased rapidly in recent years and reduced treatment options, particularly for *Neisseria gonorrhoeae*. Most prevalent incurable sexually transmitted infections are the hepatitis B virus (HBV), herpes simplex virus (HSV), human immunodeficiency virus (HIV), and human papillomavirus (HPV). HPV infection causes 0.5 million cases of cervical cancer and 0.27 million cervical cancer deaths annually (WHO). Effective and safe vaccines are currently available for HBV and HPV.

The majority of STIs have no symptoms or display only mild symptoms, significantly inhibiting diagnosis and promoting their spread. In several cases, undiagnosed and uncured STIs can lead to serious reproductive health consequences such as infertility in the case of *Chlamydia trachomatis* and *Neisseria gonorrhoeae*, mother-to-child transmission, and adverse birth outcomes. STI infections in pregnant women can lead to low birth weight and prematurity, stillbirth, neonatal death, pneumonia, sepsis, and neonatal conjunctivitis. In addition, several STIs, such as HSV and syphilis, can increase the risk of HIV acquisition. Thus, sexually transmitted infections are a major health concern worldwide.

1.1.1. Epidemiology and pathology of *Chlamydia trachomatis* infection

Chlamydia trachomatis can be considered the most prevalent sexually transmitted infection worldwide. In 2012, the global estimates of *C. trachomatis* infections among women and men aged 15–49 years were 4.2% and 2.7%, respectively. These figures correspond to an estimated 131 million new cases of *C. trachomatis* annually (Newman et al., 2015).

The estimated prevalence of *C. trachomatis* in Estonia is 6.9% and 2.7%, respectively, among women and men aged 18–35 years (Uusküla et al., 2008). *C. trachomatis* prevalence can be in particularly high among sexually active young adults. A study of 15–25 year old young adults (N=650) visiting Sexual Health Clinique in Tartu, Estonia in 2014 resulted in 14.9% and 11.7% *C. trachomatis* infection rates among women and men, respectively (Jevtuševskaja et al., 2016).

In most cases, *C. trachomatis* infection remains asymptomatic. It is estimated that 70–80% of women with *C. trachomatis* infection do not display any symptoms (Jevtuševskaja et al., 2016). However, in some cases, infection with *C. trachomatis* can result in acute salpingitis and pelvic inflammatory disease (PID), causing such long-term consequences as chronic pain, ectopic pregnancy, and infertility. In addition to sexual contact, *C. trachomatis* can be also transmitted from mother to newborn during vaginal delivery, causing conjunctivitis and pneumonia in the newborn. Ocular *C. trachomatis* can cause trachoma, the leading cause of blindness worldwide. Recent findings have also revealed a strong depletion of the p53 tumor suppressor during *C. trachomatis* infection, suggesting a potential pro-carcinogenic effect of the infection (González et al., 2014).

C. trachomatis is divided into several serovars that are associated with different clinical manifestations. Serovars A-C are associated with eye infections causing conjunctivitis and trachoma. These infections are a major health concern in remote areas of Africa and Asia (Taylor et al., 2014).

C. trachomatis infection of the genital tract is generally caused by serovars D-K. These serovars can also cause conjunctivitis, pharyngitis, and pneumonia in newborns when transmitted perinatally (Darville, 2005).

Whereas serovars A-K are generally confined to the mucosal epithelium, *C. trachomatis* lymphogranuloma venereum (LGV) serovars L1-L3 can cross the epithelium and spread to the lymphatics. LGV serovars are relatively rare in the U.S. and Europe but are particularly prevalent in tropical and subtropical areas of developing countries.

C. trachomatis infection can be treated with antibiotics such as zithromycin or doxycycline. The infection can also be cleared spontaneously in the absence of treatment as a consequence of adaptive and innate immune responses (Geisler, 2010).

C. trachomatis infect epithelial cells of the urogenital tract and conjunctiva. Epithelial host cells are the primary initiators and propagators of the host immune response to *C. trachomatis* infection. Infected epithelial cells secrete chemokines and cytokines, recruiting inflammatory leukocytes to the site of infection and inducing cellular inflammatory response. These mediators induce direct damage to the host epithelial tissue, causing scarring of the tissue that ultimately leads to inflammatory pathologic conditions like PID, particularly upon repeated infections (Darville and Hiltke, 2010).

1.1.2. Microbiology and molecular biology of *C. trachomatis*

C. trachomatis is an obligate intracellular Gram-negative bacterial pathogen. *C. trachomatis* has a biphasic developmental cycle. A small and metabolically inactive elementary body attaches and enters epithelial cells. In the host cell, it transfers to a larger metabolically active reticulate body that replicates by binary fission. Prior to host-cell lysis and release, the reticulate bodies transform into the elementary bodies.

C. trachomatis has a 1Mbp chromosome and a multicopy 7.5kb plasmid (Zhong, 2017). Although the plasmid is not required for *C. trachomatis* growth *in vitro*, it has been shown to be an important virulence factor required for induction of genital tract infection in mouse models (O'Connell et al., 2007, Farencena et al., 1997). *C. trachomatis* cells carry on average 4–10 copies of the cryptic plasmid, depending on the strain and development cycle stage (Pickett et al., 2005, Seth-Smith et al., 2009).

The plasmid encodes eight open reading frames or plasmid glycoproteins pGP1-8 (Zhong, 2017). Sequence homology data indicated that pGP7 and pGP8 are probably involved in plasmid replication and pGP1 is homologous to double-stranded DNA helicase. Sequence data also showed that pGP5 and pGP6 could be involved in regulating plasmid copy number and partitioning (Thomas et al., 1997). Deletion analysis showed that pGP1, pGP2, pGP6, and pGP8 are important for plasmid maintenance (Gong et al., 2013, Liu et al., 2014a, Song et al., 2013). pGP3 is a major factor involved in plasmid-dependent pathogenicity and induction of host inflammatory response (Liu et al., 2014b, Ramsey et al., 2014). pGP4 is a transcriptional regulator of pGP3 and several chromosomal genes, like glycogen synthase glgA, which is likely to be important for chlamydial virulence (Song et al., 2013). pGP5 is a negative regulator for several plasmid-dependent genes (Liu et al., 2014a). Thus, pGP4 and pGP5 are probably involved in chlamydial adaptation to various animal tissue environments.

1.2. Molecular diagnostics of *C. trachomatis*

Several STIs are considered to be majorly asymptomatic, including *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Mycoplasma genitalium*, *Mycoplasma hominis*, *Ureaplasma sp.* (Ona et al., 2016, Takahashi et al., 2006). The lack of correlation between symptoms and these STI infections makes them difficult to diagnose based on clinical observation. Routine screening of high-risk groups could control the spread of these infections and prevent their long-term consequences, such as infertility and PID. This requires the support of accurate and time-efficient diagnostics.

Several different molecular diagnostics techniques are applied for specific pathogen detection. In general, *C. trachomatis* can be diagnosed using culture, nucleic acid amplification techniques (NAAT), or immumoassays, which are explained below.

1.2.1. Specimen type and collection for *C. trachomatis* diagnostics

The accuracy of the diagnostic test is highly affected by specimen quality. Several sources have reported reduced sensitivity due to inappropriate specimen

collection or transportation. Culture-based and other diagnostics methods focusing on detection of live pathogens are particularly sensitive towards specimen collection and handling, causing an up to 30% reduction in sensitivity due to inadequate specimens (Black, 1997).

Several sample types have been used for STI diagnostics, including urine, semen, swab, and tissue samples from relevant anatomical sites. To date, the FDA has approved the use of urine, female vaginal and endocervical swabs, and male urethral swabs for *C. trachomatis* detection (Prevention, 2014).

Specimens with less invasive collection types are preferred, and self-collected vaginal swabs and urine samples are accepted for STI diagnostics. Urine specimens are accepted by all FDA accredited NAAT-based diagnostics methods. In addition to their non-invasive and self-collected nature, urine samples tolerate prolonged storage at RT°C (Prevention, 2014). Until recently, first void urine (FVU) samples were accepted for *C. trachomatis* diagnostics only in males. Now urine specimens are accepted for STI diagnostics for both males and females, and no timing of specimen collection is required, with midstream urine proven acceptable for NAAT diagnostics of *C. trachomatis* (Mangin et al., 2012, Prevention, 2014).

1.2.2. Culture-based techniques for *C. trachomatis* diagnostics

C. trachomatis culture detects viable infectious organisms. The culture is performed by inoculating a cell culture monolayer (e.g. HeLa 229) with patient swab specimens. If sufficiently viable *C. trachomatis* is present in the sample, it will infect the cells and the intracytoplasmic inclusions will be observed after 48–72 h. Gram, Giemsa, or iodine staining can be used; however, these stains are less suitable for specific chlamydial inclusion detection, and fluorescently labeled antibodies are better to apply. The antibodies target chlamydial lipopolysaccharide (LPS) to recognize all chlamydial species or major outer membrane proteins (MOMP) for specific *C. trachomatis* detection. Sonication is often applied prior to inoculation to disrupt intracellular inclusion bodies and separate elementary bodies (Black, 1997).

C. trachomatis culture was considered the gold standard more than 20 years ago. The culture method is 100% specific and is still applied in some cases where high specificity is required or when a viable organism needs to be obtained for further studies, such as antibiotics susceptibility or genotyping. Another advantage of culture methods is their low contamination rate.

The clinical sensitivity of the culture is at best 60–80% that of NAAT and depends highly on the experience of the technicians performing the procedure (**Figure 1**) (Meyer, 2016). Culture-based methods have stringent sample collection and transport requirements in order to preserve viable *C. trachomatis*. The other factors commonly affecting culture sensitivity are toxic substances present in clinical specimens and overgrowth of the cell culture by commensal

microorganisms. Availability of the *C. trachomatis* culture is also considered limited to clinicians due to its expense, the high level of technical expertise required, and lengthy turn-around time (3–7 days) (Black, 1997).

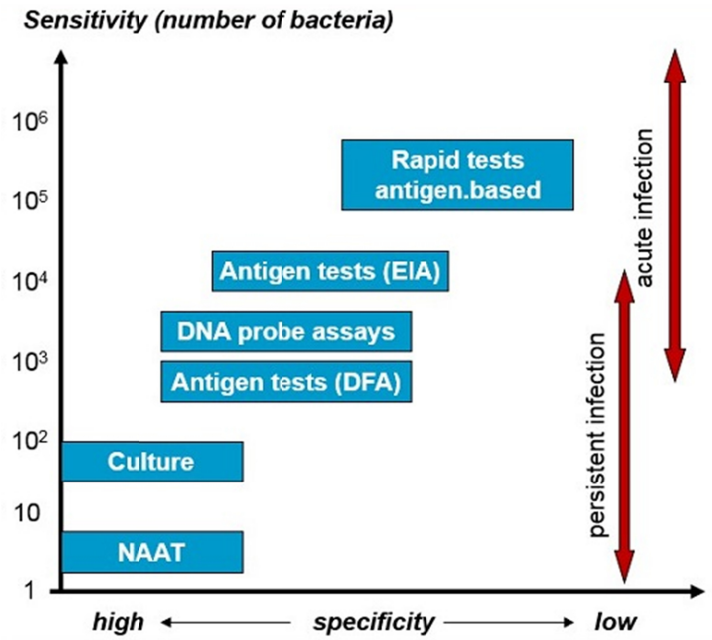

Figure 1. Relative sensitivity and specificity of techniques used to diagnose *C. trachomatis* (Meyer, 2016). Sensitivity is described as the minimum number of bacteria detected by the test. Highly sensitive tests require less pathogen for detection. The pathogen amount in the sample during acute (primary) and persistent (longer lasting) infections is also indicated. EIA – Enzyme Immunoassay; DFA – Direct Immunofluorescence Assay; NAAT – nucleic acid amplification techniques.

1.2.3. NAAT-based tests for *C. trachomatis* diagnostics

Nucleic acid amplification-based techniques are very sensitive and specific methods and are therefore considered a new gold standard for several pathogen diagnostics, including *C. trachomatis*. In contrast to culture, NAAT tests do not depend on viable pathogens; therefore, they have less stringent sample type, collection, and handling requirements. Furthermore, due to improvements to NAAT, less invasive sample types can be used for pathogen diagnostics. Due to the high sensitivity of NAAT, contamination caused by product carryover can be a problem, though.

Polymerase chain reaction (PCR) is the most common NAAT. It has been applied for *C. trachomatis* detection since 1993 and is still applicable in several FDA-approved methods, such as Abbott RealTime, Cepheid Expert, and

Amplicor CT/NG tests (**Table 1**). Most of the PCR-based NAAT use fluorescently labelled oligonucleotide probes for specific amplification product detection. NAAT assays offer high sensitivity, usually well above 90%, and specificity of ≥ 99% (**Figure 1**).

Table 1. FDA approved NAAT tests for *C. trachomatis* diagnostics (Prevention, 2014)

Test name (manufacturer)	**NAAT**	**Amplification target**
Abbott RealTime CT/NG (Abbott Molecular Inc.)	PCR	Two distinct regions of the cryptic plasmid
BD ProbeTec ET and Qx assays (Becton Dickinson)	SDA	Cryptic plasmid target
Xpert CT/NG assay (Cepheid)	PCR	Chromosomal region
Aptima COMBO 2 assay (Hologic)	TMA	23S rRNA
Amplicor and cobas CT/NG test (Roche Molecular Diagnostics)	PCR	Cryptic plasmid and chromosomal target

Cryptic plasmid is a commonly used target for *C. trachomatis* NAAT, as it is present in several copies per cell. rRNA genes are also used for NAAT assays, as they may be present in up to 15 copies per bacterial genome (Klappenbach et al., 2001). However, rRNA genes can be relatively conserved among closely related species, possibly affecting specificity levels of the assay.

As an intracellular pathogen, *C. trachomatis* is considered to be highly conserved and genetically stable. However, in 2006, a 377 bp deletion of the cryptic plasmid coding sequence 1 (CDS1) region was discovered in Sweden (and is known as the Swedish variant) (Unemo and Clarke, 2011). The deletion overlapped with the *C. trachomatis* single target NAAT assays used by Abbot and Roche diagnostics systems at the time. This caused rapid spread of the Swedish variant due to thousands of false negative diagnoses. Since then, dual-targeting has often been applied with an additional chromosomal target (ompA). The Swedish variant case stresses the importance of target selection for NAAT diagnostics assays.

1.2.4. Non-culture, non-NAAT tests for *C. trachomatis* detection

Other diagnostics tests for *C. trachomatis* include antigen-based detection methods (DFA, EIA), DNA probe assays, and serological tests.

DFA (Direct Immunofluorescence Assay) is based on direct microscopic visualization of specimens by staining with a *C. trachomatis*-specific fluorescent antibody. MOMP- and LPS-targeting antibodies are often used. DFA tests have high specificity but are generally less sensitive than culture-based methods (**Figure 1**) (Black, 1997). In addition, although DFA staining is rapid, microscopic evaluation of each sample is laborious and requires highly experienced personnel.

EIA (Enzyme Immunoassay) is based on immunochemical detection of chlamydial LPS with enzyme-linked antibodies. The product of the enzymatic reaction, after specific antibody binding to the antigen, is then detected with a spectrophotometer or fluorescence reader. LPS is often used in EIA as an antigen since it is more abundant and soluble than MOMP. Although LPS is considered a genus-specific antigen, some cross-reactivity with other Gram-negative bacteria has been observed (Haralambieva et al., 2001). Some EIAs also do not differentiate between different chlamydia species (Jones et al., 2003). Thus, relatively low specificity is a problem for EIA (**Figure 1**).

C. trachomatis can also be detected by hybridization of the DNA probe to the unamplified genomic DNA. The test uses a chemiluminescent DNA probe, which hybridizes to a chlamydia-specific sequence of the 16S rRNA. DNA-rRNA hybrid produces a luminometer-detectable chemiluminescent response. Actively dividing *C. trachomatis* contain up to 10^4 copies of 16S rRNA; thus, DNA probe assay has higher sensitivity than EIA (81% and 50% respectively, as compared to NAAT) (**Figure 1**) (Lauderdale et al., 1999). DFA, EIA, and DNA probe hybridization tests are no longer recommended for routine *C. trachomatis* diagnostics due to their insufficient accuracy (**Figure 1**) (Prevention, 2014).

Serological tests are generally not useful for *C. trachomatis* detection, as the antibody response becomes detectable only after several weeks, and antibody titers may be low. Additionally, antibodies elicited by the infection are long lived and a positive test will not distinguish between previous and current infections. Although serology is considered to be helpful in some cases of chronic infections with low pathogen counts and for diagnostics of LGV serovar infections due to stronger antibody responses of the systemic disease, it is not widely applied (Meyer, 2016, Black, 1997).

1.3. Point-of-care (POC) diagnostics

Globally, control of curable STIs is increasingly focusing on point-of-care tools. POC tests can be defined in multiple ways. Generally, any diagnostic tool that can provide accurate results and facilitate treatment within the same clinical visit is considered POC applicable. However, POC test requirements are often designed based on the ASSURED criteria: Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free, and Deliverable to end-users.

The purpose of POC testing is to reduce the time-to-result, allowing STI diagnosis to be made within the same visit to the doctor. This is particularly important in resource-poor environments where low patient follow-up rates are reported, as patients are often unable to return to the clinic to receive the appropriate treatment (Schwebke et al., 1997). This causes the spread of infection to sex partners, transmission from mother to child, and serious health consequences for patients. Syndromic management of STIs is majorly inapplicable due to the asymptomatic nature of most infections and can lead to inappropriate treatment, contributing to the growing global problem of antibiotic resistance. Therefore, appropriate POC testing is required to control the spread and health issues resulting from STIs.

In addition to reducing time to treatment, POC offers also a cost-efficient strategy for large-scale screenings and is often the only possibility for appropriate diagnostics in resource-constrained regions, where electricity, pure water, or skilled personnel are simply unavailable.

Most of the POC tests are immunochromatographic assays that detect pathogen antigens using lateral-flow technology. However, poor accuracy of these tests is a concern that has limited their wider application (**Figure 1**). Currently, only HIV and syphilis POC tests have been accepted for diagnostics applications, while others struggle to produce sensitivity and specificity that meets the laboratory level diagnostics of the STI.

The OraQuick POC test for HIV detection at home was approved by the FDA in 2012. The test detects HIV-1 and HIV-2 antibodies from oral swab in 20 min with 93% sensitivity and close to 100% specificity (Tucker et al., 2013). Several *Treponema palladium* antibody tests from whole blood, serum, or plasma are available for fast syphilis diagnostics. The sensitivity of the syphilis POC tests from whole blood ranges from 86.3 to 100%, and their specificity is in the range of 95.9%–97.0% (Cristillo et al., 2017).

1.3.1. POC diagnostics of *C. trachomatis*

Several assays for *C. trachomatis* detection are considered POC applicable, although none of them can currently fully meet the ASSURED criteria.

Gram staining of urethral smears is a quick assay that involves microscopic evaluation of stained urethral samples. Large-scale evaluation involving 26,000 male patients has shown relatively good sensitivity values of 91.0% for sympto-

matic male patients and 83.8% for all male patients irrespective of symptoms (**Table 2**) (Bartelsman et al., 2015). However, method specificity values were poor at 53.1% and 74.1%, respectively (**Table 2**) (Bartelsman et al., 2015). Additionally, Gram staining has been criticized due to its uncomfortable/painful sample collection, the requirement of skilled personnel for microscopic evaluation, and poor consistency of results (Apoola et al., 2011, Smith et al., 2003).

Urine flow cytometry could potentially be applied, as increased white cell counts in first void urine samples have been observed for *C. trachomatis*-infected patients. Although the test has relatively good sensitivity (93.7%), it fails to distinguish *C. trachomatis* and *Mycoplasma genitalium* infections, which have different treatment profiles (**Table 2**) (Pond et al., 2015). Due to the robust and rapid nature of the test, it could be useful for the pre-screening purposes.

The Handilab C test detects the *C. trachomatis*-specific enzyme Peptidase 123A. The test is rapid and robust, does not require additional instrumentation, and therefore meets several POC criteria. However, its sensitivity is quite poor (22.5%) (**Table 2**) (van Dommelen et al., 2010).

The majority of the *C. trachomatis* POC assays to date are immune chromatographic tests based on lateral-flow-technology that detect chlamydia-specific LPS antigen in a biological sample. The Biorapid, ACON, and QuickVue *C. trachomatis* tests are applicable in POC settings and have relatively high specificity levels (**Table 2**) (Nuñez-Forero et al., 2016, Hurly et al., 2014, van Dommelen et al., 2010). However, the sensitivity of the immunochromatographic tests remains in the low range of 20–40% according to large-scale studies (**Table 2**) (van Dommelen et al., 2010, Nuñez-Forero et al., 2016).

Attempts have been made to increase antigen-based assay sensitivity. In the newly developed aQcare assay, europium-chelated nanoparticles and a time-resolved fluorescence (TRF) reading system were applied for more accurate results. The aQcare system requires a small portable signal-acquisition device for application and has shown a significantly increased sensitivity levels compared to ACON and QuickVue assays (**Table 2**) (Ham et al., 2015). However, additional evaluation by other research groups is required to support this data.

Table 2. Rapid tests for *C. trachomatis* detection

Method	Description	Sensitivity (95% CI)	Specificity (95% CI)
Gram-stained urethral smear	Microscopic evaluation of the Gram-stained urethral swab sample	83.8% (81.2–86.1%) (men, urethral swab) (Bartelsman et al., 2015)	74.1% (73.0–75.2%) (men, urethral swab) (Bartelsman et al., 2015)
		91.0% (89.5–92.3%) (symptomatic men, urethral swab) (Bartelsman et al., 2015)	53.1% (51.8–54.4%) (symptomatic men, urethral swab) (Bartelsman et al., 2015)
Urine flow cytometry	Automated flow cytometry of the first void urine for white blood cell count determination	93.7% (67.7–99.6%) (symptomatic men, urine) (Pond et al., 2015)	NA
Handilab-C test	Detects *C. trachomatis*-specific enzyme Peptidase 123A	22.5% (13.0–31.7%) (women, vaginal swab) (van Dommelen et al., 2010)	88.9% (86.4–91.3%) (women, vaginal swab) (van Dommelen et al., 2010)
Biorapid Chlamydia Ag test	Immunochromatographic test for the detection of *C. trachomatis*-specific antigen	17.1% (8.9–25.2%) (women, vaginal swab) (van Dommelen et al., 2010)	93.7% (91.9–95.5%) (women, vaginal swab) (van Dommelen et al., 2010)
ACON Chlamydia rapid tests	Immunochromatographic test for the detection of *C. trachomatis*-specific antigen	22.7% (2.9–42.5%) (ACON CT, symptomatic women, endocervical swab) (Nuñez-Forero et al., 2016)	100% (99.7–100%) (ACON CT, symptomatic women, endocervical swab) (Nuñez-Forero et al., 2016)
		30.5% (17.9–43.1%) (ACON Duo, symptomatic women, endocervical swab) (Nuñez-Forero et al., 2016)	99.8% (99.2–100%) (ACON Duo, symptomatic women, endocervical swab) (Nuñez-Forero et al., 2016)

Method	Description	Sensitivity (95% CI)	Specificity (95% CI)
		43.8% (19.8–70.1%) (men, urine) (Hurly et al., 2014)	98.3% (93.9–99.8%) (men, urine) (Hurly et al., 2014)
		66.7% (22.3–95.7%) (women, vaginal swab) (Hurly et al., 2014)	91.3% (82.0–96.7%) (women, vaginal swab) (Hurly et al., 2014)
QuickVue Chlamydia rapid test	Immunochromatographic test for the detection of *C. trachomatis*-specific antigen	37.74% (23.7–51.7%) (symptomatic women, endocervical swab) (Nuñez-Forero et al., 2016)	99.4 (98.6–100%) (symptomatic women, endocervical swab) (Nuñez-Forero et al., 2016)
		25.0% (15.7–34.3%) (women, vaginal swab) (van Dommelen et al., 2010)	99.7% (99.3–100%) (women, vaginal swab) (van Dommelen et al., 2010)
aQcare Chlamydia TRF kit	Lateral-flow immunoassay (LFIA) that uses europium-chelated nanoparticles and a time-resolved fluorescence reader	88.2% (67.4–97.7%) (urine) (Ham et al., 2015)	94.7% (90.1–96.9%) (urine) (Ham et al., 2015)
		93.8% (88.6–97.0%) (endocervical and urethral swabs) (Ham et al., 2015)	96.8% (94.8–98.1%) (endocervical and urethral swabs) (Ham et al., 2015)
Chlamydia rapid test (Diagnostics for the Real World)	Signal Amplification System (SAS)-based immunochromatographic test	41.4% (23.5–61.1%) (men, urine) (Hurly et al., 2014)	89.0% (82.2–93.8%) (men, urine) (Hurly et al., 2014)
		74.2% (61.5–84.5%) (women, vaginal swab) (Hurly et al., 2014)	95.7% (91.3–98.2%) (women, vaginal swab) (Hurly et al., 2014)

Method	Description	Sensitivity (95% CI)	Specificity (95% CI)
		41.2% (31.9–50.9%) (women, vaginal swab) (van der Helm et al., 2012)	96.4% (95.0–97.5%) (women, vaginal swab) (van der Helm et al., 2012)
Cepheid GeneXpert CT/NG	Automated sample preparation and RT-PCR amplification system	98.7% (93.1–100%) (women, vaginal swab) (Gaydos et al., 2013)	99.4% (98.9–99.7%) (women, vaginal swab) (Gaydos et al., 2013)
		97.4% (91.0–99.7%) (women, endocervical swab) (Gaydos et al., 2013)	99.6% (99.1–99.8%) (women, endocervical swab) (Gaydos et al., 2013)
		97.6% (91.5–99.7%) (women, urine) (Gaydos et al., 2013)	99.8% (99.5–100%) (women, urine) (Gaydos et al., 2013)
		97.5% (91.4–99.7%) (men, urine) (Gaydos et al., 2013)	99.9% (99.6–100%) (men, urine) (Gaydos et al., 2013)

* Unless stated otherwise, patients enrolled to the study were not discriminated based on their symptoms.

A signal amplification system (SAS) is another technology used to increase immunoassay sensitivity by applying a multiple hapten-labelled antibody for stronger visual signal when detecting a protein target (applied by the Diagnostics for the Real World). The test has shown somewhat increased sensitivity; however, clinical evaluation results are quite variable and depend on the sample type (**Table 2**) (van der Helm et al., 2012, Hurly et al., 2014). Therefore, the effect SAS technology on the clinical performance of the immunoassay is unclear.

Cepheid employed NAAT in a fully automated system that enables pathogen detection within 90 min. The GeneXpert system uses cartridges that combine on-board sample preparation with real-time PCR. Cepheid GeneXpert has sensitivity and specificity that meet laboratory level NAAT diagnostics and is applicable with different sample types (**Table 2**) (Gaydos et al., 2013). However, the analysis requires the use of thermal and optical module instrumentation.

To conclude, to date there is no test for *C. trachomatis* detection that meets all ASSURED criteria. Immunochromatography-based tests are very fast, robust, affordable, and instrument free; however, their sensitivity levels in clinical evaluations are poor. Automated systems with NAAT-based detection offer excellent clinical performance levels but require use of appropriate instrumentation. The speed and affordability of these tests are also questionable in terms of POC application. Therefore, new strategies must be explored with one opportunity being the use of isothermal NAAT. These are more robust and faster and have less stringent instrumentation requirements than PCR and could potentially offer laboratory level sensitivity.

1.4. Isothermal nucleic acid amplification

Isothermal techniques enable nucleic acid amplification at constant temperatures and therefore do not require temperature cycling as conventional PCR does. Therefore, isothermal amplifications have strong advantages for POC applications. Since emerging in the 1990s, several methods have been developed that can be classified into four categories (Niemz et al., 2011):

- RNA transcription-based (NASBA, TMA)
- DNA replication-based with enzymatic duplex melting/primer annealing (HDA, RPA)
- DNA polymerase-mediated strand displacement-based methods form linear or circular targets (RCA, LAMP)
- Polymerase extension/strand displacement-based plus single strand cutting event (SDA, NEAR)

Nucleic acid sequence-based amplification (NASBA) and **transcription-mediated amplification (TMA)** use a retroviral strategy for RNA replication via cDNA intermediate (**Figure 2a**) (Compton, 1991). These methods are particularly useful for RNA virus diagnostics. They use two sequence-specific primers, reverse transcriptase, DNA dependent RNA polymerase, and in some cases RNase H. Although reaction is performed at 41°C, it does require a 65°C preheating step for initial primer annealing (Compton, 1991).

Helicase-dependent amplification (HDA) mimics DNA replication fork and uses helicase to separate double-stranded DNA (**Figure 2b**) (Vincent et al., 2004). Originally, HDA used *E. coli* UvrD helicase, SSB protein to prevent unwound DNA from re-annealing and DNA polymerase I Klenow fragments. The reaction can be successfully performed with the use of two primers at 37°C. **Recombinase polymerase amplification (RPA)** uses recombinase to introduce primers to the template strand, SSB to stabilize ssDNA structures formed during amplification, and a DNA polymerase with strand-displacement activity (*Bacillus subtilis* Pol I, Bsu) (**Figure 2b**) (Piepenburg et al., 2006). RPA reaction is performed at 37°C–42°C.

Rolling circle amplification (RCA) employs a rolling circle mechanism used for replication of plasmids and viral genomes. RCA uses highly processive DNA polymerase from Phi29 bacteriophage to synthesize multiple repetitions of the circular template (**Figure 2c**) (Fire and Xu, 1995). **Loop-mediated isothermal amplification (LAMP)** is another method using strand displacement and cyclic amplification (**Figure 2c**). LAMP reaction is performed at around 63°C because at this temperature dsDNA is in a dynamic equilibrium state that allows primers to anneal to the template without the denaturation step. LAMP reaction is initiated with a non-cycling amplification step that results in a single-stranded double stem–loop DNA structure or dumbbell structure (Notomi et al., 2000). The latter allows the reaction to enter the cycling amplification, resulting in a high molecular weight product. LAMP uses four primers, although two additional loop primers can be added to increase the sensitivity or speed of the reaction (Nagamine et al., 2002).

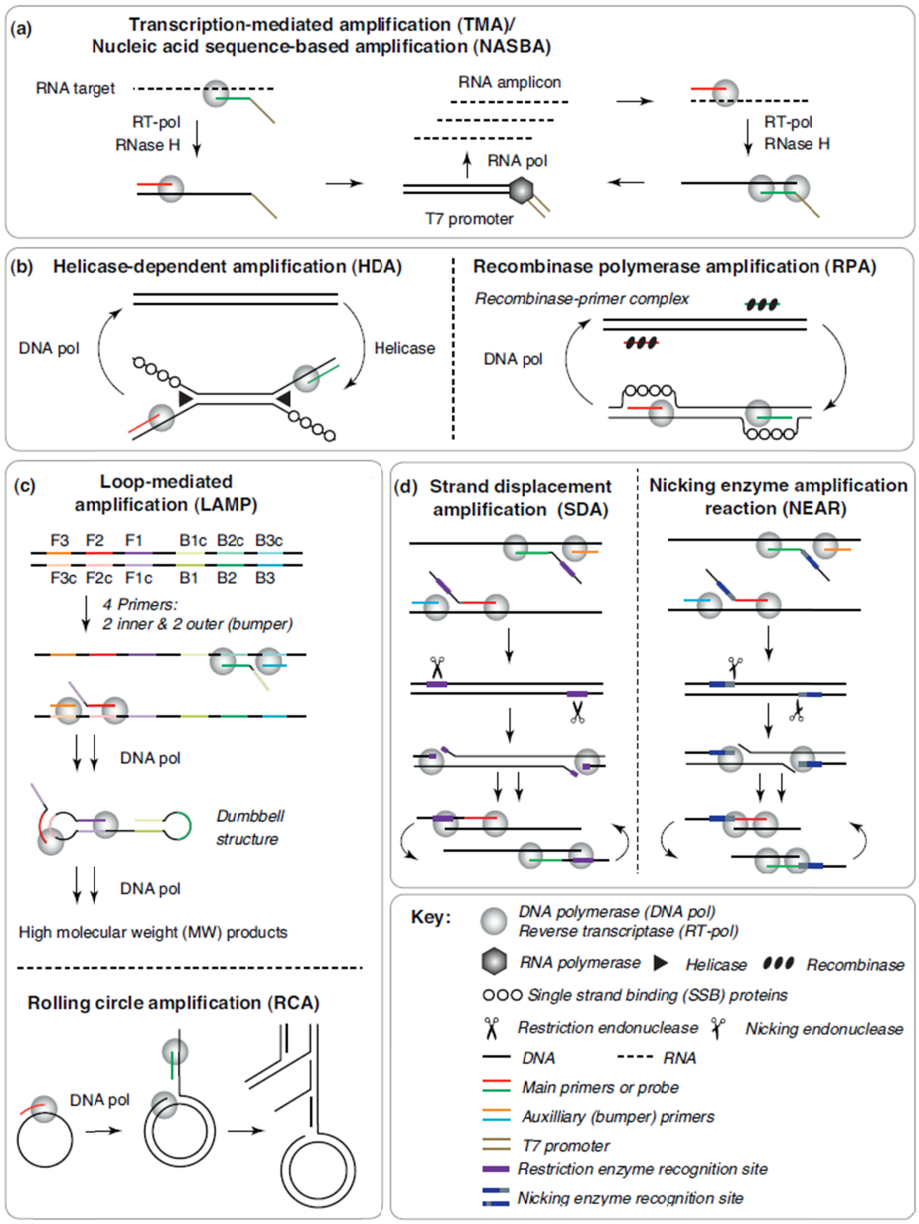

Figure 2. Overview of isothermal nucleic acid amplification reactions (modified from (Niemz et al., 2011). (a) Methods based on RNA; (b) Methods based on DNA replication with enzymatic duplex melting/primer annealing; (c) Methods based on strand-displacing polymerization from linear or circular targets; (d) Methods based on a polymerase extension and a single-strand cutting event.

Strand displacement amplification (SDA) uses a restriction endonuclease to nick the target DNA strand and exonuclease-deficient DNA polymerase to displace DNA strand from the nick site (**Figure 2d**) (Hellyer and Nadeau, 2004). Phosphothioates are incorporated into the amplicon during amplification so that the endonuclease only cuts one strand. SDA requires an initial denaturation step, but the rest of the reaction is performed at 37°C. **Nicking enzyme amplification reaction (NEAR)** uses the single-strand nicking endonuclease N.BstNB and therefore does not require modification of the amplicon strand (**Figure 2d**) (Morgan et al., 2000). Additionally, NEAR uses thermophilic enzymes that allow forgoing the initial denaturation step, and the reaction can be performed as a single-step procedure at 54–60°C.

Several isothermal amplification techniques have been applied commercially for STI diagnostics. Hologic uses a TMA-based system that can be employed for *C. trachomatis, N. gonorrhoeae, Trichomonas vaginalis, Mycoplasma genitalium,* Zika virus, HBV, HCV, HSV, HIV and HPV diagnostics. bioMérieux implement NASBA and Becton Dickins SDA for nucleic acid-based diagnostics. RPA is commercialized by TwistDX, which currently offers several food-borne pathogen detection kits. Eiken's LAMP-based Loopamp technology is applied for several food and environmental pathogen-detection purposes as well as *Mycobacterium tuberculosis* and *Plasmodium* species detection for tuberculosis and malaria diagnostics, respectively.

1.4.1. Loop-mediated isothermal amplification (LAMP)

LAMP was developed in 2000 and remains the most widely applied isothermal amplification technique to date. LAMP is a highly efficient and specific amplification, its primers recognizing six distinct regions in the target DNA. However, non-template amplification (amplification of primers without template DNA) can be a concern, particularly with multiplexed reactions, and non-specific amplification can be observed upon prolonged incubation (Tanner et al., 2012, Francois et al., 2011).

During LAMP reaction, a large amount of DNA is synthesized that has a ladder-like appearance on the agarose gel. This is a unique feature of LAMP, as reaction results can be evaluated by the naked eye due to formation of white turbidity by magnesium ions and pyrophosphates (Mori et al., 2001). In addition, a fluorescent metal indicator, like calcein, can be added to the reaction, generating substantial naked-eye visible change in the fluorescence upon product formation (Tomita et al., 2008), a great advantage for POC applications.

Because of the aforementioned characteristics, LAMP has been widely applied for end-point as well as real-time detection of pathogen RNA/DNA, including bacteria (Hanaki et al., 2011, Wang et al., 2011, Su et al., 2014, Kaewphinit et al., 2013, Gelaw et al., 2017), viruses (Kumvongpin et al., 2017, Singleton et al., 2014, Priye et al., 2017, Wang et al., 2016, Ge et al., 2013), fungi (Inácio et al., 2008), protozoan parasites (Yongkiettrakul et al., 2014,

Adao and Rivera, 2016), and nematodes (Mugambi et al., 2015). LAMP has also been applied for genetically modified material identification (Xu et al., 2013), SNP analysis (Lu et al., 2017, Zhang et al., 2016), gene copy number discrimination (Nakamura et al., 2010), and RNA expression analysis (Muto et al., 2011).

Due to the principle of oligonucleotide annealing, LAMP has relatively stringent incubation temperature requirements and needs incubation within 57–67°C for assay performance (Francois et al., 2011).

Several options have been investigated for instrument-free POC application of LAMP, including electricity-free heaters based on exothermic chemical reactions and appropriate phase change materials for successful incubation (LaBarre et al., 2011). Disposable pocket warmers have been applied as a heat source required for reaction (Hatano et al., 2010, Hatano et al., 2011).

1.4.2. Recombinase polymerase isothermal amplification (RPA)

RPA is a relatively new method that relies on an enzymatically driven primer binding process rather than the physio-chemical dependent hybridization used in LAMP. For that reason, RPA can tolerate ambient reaction temperatures (31°C–43°C) and can therefore be applied with a simple non-instrumented heat source such as a chemical heater, allowing lowering of the ambient temperature as low as 10°C (Lillis et al., 2014). This makes RPA a particularly attractive candidate for POC applications. However, for optimal reaction time and sensitivity, RPA temperatures should be kept at 37–42°C (**Figure 3**), at which reaction time required to result can be 3–12 min depending on the template amount (Wang et al., 2017).

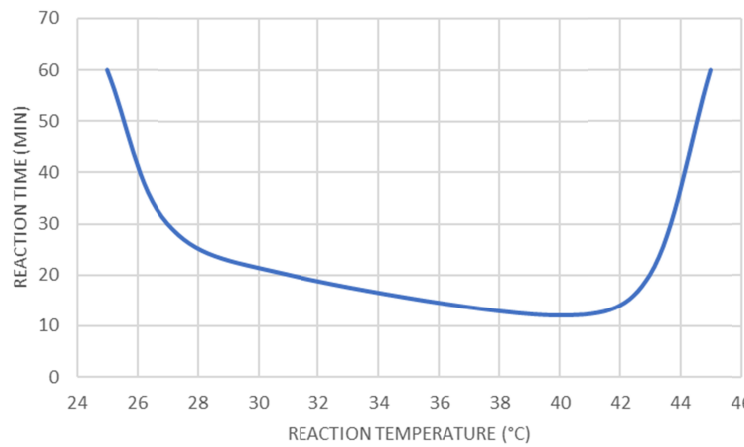

Figure 3. RPA reaction time dependence on incubation temperature at template concentrations near LOD (based on data obtained from TwistDX, (Lillis et al., 2014, Wang et al., 2017). Required reaction time for product formation is plotted against reaction temperature.

RPA reaction uses crowding agents like high molecular weight polyethylene glycol (PEG) to improve amplification efficiency. However, due to the viscous nature and relatively low amplification temperature of PEG, localized depletion of the amplification reagents occurs, restraining amplification cascade. Thus, a mixing step after 3–6 min of incubation is required for optimal performance of RPA (Lillis et al., 2016).

RPA is also an attractive candidate for POC applications because its reagents are supplied in a lyophilized form and are relatively stable, with no reduction of the assay performance after 12 weeks' storage at 25°C and 6 weeks' storage at 45°C (Lillis et al., 2016).

1.5. Clinical sample preparation prior to nucleic acid amplification

A major obstacle for instrument-free NAAT application for POC diagnostics is the requirement of biological sample preparation prior to amplification. Nucleic acid purification is widely applied in the laboratory settings, as it enables extraction of DNA/RNA from cellular material and eliminates the majority of amplification inhibitors. Although some attempts have been made in the form of single-use sample-prep devices, sample preparation remains an unresolved issue in POC diagnostics.

1.5.1. Biological sample substances associated with NAAT inhibition

Biological sample inhibitors are a major concern for molecular diagnostics, significantly affecting downstream amplification efficiency, increasing false-negative results, and causing loss in sensitivity and accuracy of diagnostic assays.

Studies have shown that 7% and 11.9% of urine samples were inhibitory for PCR and TMA, respectively. PCR inhibition was associated with beta-human chorionic gonadotropin (beta-HCG) and salt crystals. TMA inhibition was due to hemoglobin, nitrites, and crystals (Mahony et al., 1998). Urea has also been known to inhibit PCR reaction at concentrations of 50 mM and above (Khan et al., 1991), possibly due to denaturation of the polymerase (Wilson, 1997, Schrader et al., 2012).

The presence of blood significantly inhibits PCR, as Taq DNA polymerase can tolerate 0.2% or less blood in the reaction (Abu Al-Soud and Rådström, 2000, Abu Al-Soud and Râdström, 1998). Plasma, immunoglobulin G, hemoglobin, heparin, and lactoferrin are major proposed inhibitors of blood samples (Al-Soud and Rådström, 2001, Al-Soud et al., 2000, Wilson, 1997).

Amplification inhibitors can interfere with DNA polymerase by directly binding and blocking enzyme activity. Inhibitors may also target DNA polymerase cofactor requirements, acting by reducing Mg^{2+} availability or inter-

fering with Mg^{2+} binding by the DNA polymerase. Several inhibitors interact with template DNA, preventing amplification and causing co-purification of the inhibitors. Protease and nuclease activity of the biological sample can also contribute to the reduced amplification efficiency (Wilson, 1997, Schrader et al., 2012).

1.5.2. Different sample preparation techniques applied prior to NAAT

Nucleic acid purification is the most efficient and most commonly applied method for amplification inhibitor removal. Purification is easy to apply in laboratory settings, but its cost and technical challenge make it difficult to apply in POC conditions. Therefore, several alternative sample preparation techniques have been applied that target two purposes: (i) increasing target DNA available for amplification through disruption of the cellular material and (ii) overcoming the inhibitory effect of crude biological samples.

Addition of bovine serum albumin (BSA), betaine, ssDNA binding protein (SSB) from T4 bacteriophage, or a protease inhibitors cocktail can partially reduce PCR blood inhibition. These additions allow Taq DNA polymerase to tolerate up to ten times more whole blood (up to 2%) in the PCR reaction (Kreader, 1996, Topal and Sinha, 1983, Forbes and Hicks, 1996). Heating, freeze-thawing, and diluting the sample can also increase amplification efficiency, as can activated carbon treatment (Mahony et al., 1998, Schrader et al., 2012, Verkooyen et al., 1996). Addition of mild detergents (such as Tween 20), DTT, DMSO, ß-mercaptoethanol, or PEG can also potentially reduce the action of the inhibitory substances (Schrader et al., 2012).

One of the proposed mechanisms for removal of the urine sample inhibitory substances is freeze-thawing, or overnight storage at either 4°C or –70°C. 42% and 79% of urine samples lost their inhibitory effect in PCR and TMA, respectively, after overnight storage at 4°C. –70°C storage further increased the loss of the inhibitory effect in 58% of PCR samples (Mahony et al., 1998). The mechanism of the inhibition loss is unclear, but deactivation of the labile proteinaceous inhibitors has been proposed through inducing their conformational change leading to subsequent loss of activity (Mahony et al., 1998). Alternatively, this effect might also be due to release of additional target nucleic acid molecules by disrupting microbial cells.

One of the most efficient strategies for amplification inhibition reduction is genetic manipulation of the DNA polymerase. Deletion of the N-terminal portion from Taq polymerase increases its blood tolerance by 10–100 times (Kermekchiev et al., 2009). Additional single amino acid mutation further enhances Taq tolerance towards several biological samples and inhibitors, such as blood, hemoglobin, lactoferrin, plasma, serum IgG, soil extracts, humic acid, and even DNA intercalating dyes (Kermekchiev et al., 2009). The mechanism of blood sample inhibition has been shown to be predominantly related to

reduction of DNA polymerase extension speed (Kermekchiev et al., 2009). Other researchers have also achieved increased tolerance towards several inhibitors such as heparin, humic acid, peat extract, coprolite, clay-rich soil, cave sediment, and tar by genetically modified Taq polymerase variants or chimeras (Ghadessy et al., 2001, Baar et al., 2011). Therefore, the choice of DNA polymerase has a crucial role in amplification efficiency (Wiedbrauk et al., 1995, Abu Al-Soud and Râdström, 1998).

While the issue of biological sample-associated inhibitors is mainly addressed by amplification buffer composition and DNA polymerase manipulation, several sample preparation strategies are applied for the purpose of nucleic acid release from cellular material. Sample preparation can be divided into four categories: (i) mechanical lysis, (ii) thermal treatment, (iii) enzymatic lysis, and (iv) chemical lysis.

Biological sample material can be prepared mechanically using beads mills, electrical treatment, ultrasonication, or simple freeze-thawing procedure. A major advantage of the mechanical treatment is its robustness and lack of inhibition to the downstream amplification procedure. Although attempts have been made to apply mechanical treatment in an instrument-free way, it remains difficult (Gabardo et al., 2015).

Heating is the most widely applied crude sample preparation procedure. It is simple, fast, and applicable to several biological sample types, such as blood and saliva (Poon et al., 2006, Nakanishi et al., 2011, Modak et al., 2016, Nyan et al., 2014). Boiling has been proven to be the best sample pretreatment procedure over detergent, Proteinase K, and alkali treatment for nasopharyngeal samples (Nie et al., 2012, Sun et al., 2014). However, thermal treatment is challenging to apply in POC settings due to high-temperature requirements.

Enzymatic lysis facilitates the release of nucleic acid material using bacteriolytic or proteolytic enzymes such as Proteinase K, lysozyme, PlyC, or mutanolysin. Proteinase K treatment has been applied for simulated sputum, urine, and serum samples spiked with Zika virus, for which it proved superior to heat and alkali treatment and was just as efficient as DNA extraction (Wang et al., 2016). Proteinase K has also been applied for nasopharyngeal sample treatment; however, boiling was more efficient for this sample type (Nie et al., 2012). Mutanolysin treatment has been applied for saliva samples (Nakanishi et al., 2011). PlyC lysin from the streptococcal bacteriophage was efficiently applied for Gram-positive Streptococci species (Nelson et al., 2006). Use of lysozyme is complicated by the additional requirement for favorable osmotic conditions and the sensitivity of its lysis activity towards different buffer conditions (Sedov et al., 2011). Thus, Gram-negative bacteria lysis with lysozyme requires addition of the Triton X-100 or EDTA for sufficient efficiency (Voss, 1964).

Chemical lysis includes alkali and mild detergent treatments. Alkaline lysis has been efficiently applied for nucleic acid extraction from blood sample, but sputum, urine, and other sample materials could also be suitable (Zhang et al., 1992, Rudbeck and Dissing, 1998, Wang et al., 2016). Several detergents can efficiently lyse cellular membranes. However, the majority of them are highly

inhibitory for downstream amplification due to their protein denaturing properties. Nonionic detergents such as Triton X-100 and octylthioglucoside are often applied for bacterial cell lysis (Schilling et al., 2002, Abolmaaty et al., 2000). Triton X-100 treatment was successfully applied for protozoan *Plasmodium falciparum*-infected whole blood sample treatment (Kemleu et al., 2016). The NP-40 and Tween 20 detergents have been applied for nasopharyngeal sample treatment (Nie et al., 2012).

Although several sample preparation techniques have been used, the search continues for efficient and fast lysis agents that neither interfere with downstream application nor require additional instrumentation, such as membrane-active peptides, discussed below.

1.5.3. Isothermal amplification directly from clinical samples

There are several indications that isothermal amplification techniques are less sensitive towards biological sample components than conventional PCR.

LAMP has been shown to be a relatively robust amplification technique, tolerating well pH variation within the range of 7.3–9.3 and addition of stool, urine, 10 μM hemin, 100 mM NaCl, and several anticoagulants (e.g. heparin, EDTA, and citrate) (Francois et al., 2011). LAMP has been also found to tolerate high urea concentrations of ≤1.8 M, while PCR only tolerates up to 50 mM urea (Edwards et al., 2014, Khan et al., 1991), and to be significantly less sensitive towards blood components than PCR (Poon et al., 2006, Francois et al., 2011) as well as culture medium and other biological substances such as phosphate buffered saline (PBS) (Kaneko et al., 2007).

RPA tolerates serum and hemoglobin at 50 g/l concentration; however, it is inhibited by whole blood (Kersting et al., 2014).

Several researchers have addressed the issue of isothermal amplification directly from unprocessed or minimally processed biological samples. There is growing proof that the DNA extraction step can be replaced with a simple processing procedure without significant effect on the assay's analytical and clinical sensitivity.

Proteinase K-treated Zika virus-spiked sputum, urine, and serum samples showed comparable sensitivity levels to extracted DNA (Wang et al., 2016). Triton X-100 treatment of *Plasmodium falciparum*-infected whole blood samples resulted in 85% clinical sensitivity, while extraction of the DNA increased sensitivity only to 90% (Kemleu et al., 2016).

Boiling and dilution was found to be suitable pretreatment for blood or serum samples. 95% clinical sensitivity for *Plasmodium falciparum* detection was claimed for heat-treated blood samples (Poon et al., 2006). 92% of hepatitis B virus HBV-positive clinical plasma samples were detected by simple heating procedure (Nyan et al., 2014). Crude blood lysates prepared simply by dilution in water have been shown to be sufficient for highly sensitive detection of *Plasmodium* sp by LAMP (Kongkasuriyachai et al., 2017). Additionally, heat

treatment coupled with dilution of blood and saliva samples proved to have equal efficiency to DNA extraction for *Plasmodium falciparum* detection (Modak et al., 2016).

High clinical sensitivity was detected for heat-treated respiratory samples. 87% clinical sensitivity was observed for human enterovirus detection from heat-treated nasopharyngeal swab samples (Nie et al., 2012), 90.9% sensitivity for adenovirus (ADV) detection from nasopharyngeal aspirates (Sun et al., 2014) and 70–80% clinical sensitivity for respiratory syncytial virus (RSV) detection from heat-treated respiratory samples (Hoos et al., 2017).

Unprocessed samples have been shown to be suitable for direct amplification by LAMP. The analytical sensitivity of LAMP remained the same when DNA-extracted samples were compared to direct amplification from *Klebsiella pneumoniae*-spiked clinical specimens (sputum, urine, fecal, and blood samples) and exceeded PCR sensitivity with the same samples by 10^4 times (Nakano et al., 2015). *E. coli* was also detected directly from urine samples by LAMP without any processing (Hill et al., 2008).

The choice of efficient processing procedure depends on the pathogen, sample, and isothermal amplification type. Although the potential to forgo costly and labor-intense nucleic acid purification is an intriguing possibility, further investigation is required before putting it into practice.

1.6. Antimicrobial peptides as pathogen cell lysing agents

Animals and plants use antimicrobial peptides (AMPs) as a defense mechanism against a broad range of microbes including bacteria, fungi, viruses, protozoa, and archaea (Varnava et al., 2017). Many AMPs have an amphipathic structure in which hydrophobic and cationic amino acids are clustered into spatially discrete sectors. AMPs specifically target microbes based on their cellular membrane structure, which is clearly distinguishable for a broad spectrum of microbes from multicellular animals. The outer layer of bacterial membranes exposes hydrophobic and negatively charged phospholipids (**Figure 4**), which amphipathic AMPs bind using electrostatic and hydrophobic interactions. On the other hand, the outside layer of animal cell membranes usually have no charge, and most of the negatively charged lipids are located in the inner layer facing the cytoplasm (**Figure 4**) (Zasloff, 2002). The presence of cholesterol in animal membranes has been shown to reduce AMP activity due to stabilization of the membrane (Matsuzaki et al., 1995).

Several models have been proposed for AMP antimicrobial activity. The majority of AMPs act through permeabilization and/or disruption of microbial membranes. AMPs interact with membranes due to their physicochemical properties, leading to lipid displacement and often pore formation. This leads to diffusion of the peptides into cell interior and/or collapsing of the membrane into fragments and physical disruption (or lysis) of the cell (Zasloff, 2002). The mechanism of action depends on the particular peptide. Magainin and melittin

introduce small toroidal pores into the membrane, while Cecropin P1 and MSI peptides use carpet or detergent mechanisms for membrane disruption (Li et al., 2017).

In addition to selectivity between host and microbe membranes and broad microbial action, AMPs have to act fast due to the short doubling time of bacteria (20 min in case of *E. coli*). Another required feature of AMPs is that they act through such a mechanism that bacteria cannot easily develop resistance to them (Matsuzaki, 1999).

Selectivity for microbial membranes, fast membrane-disruptive mechanism, and broad microbial action make AMPs a potentially good target for applications in clinical sample preparation prior to pathogen NAAT. Specimen treatment with membrane-active AMPs could lead to specific disruption of microbial cell membranes and facilitating release of the target nucleic acids. However, positively charged AMPs may also interact non-specifically with nucleic acids that may interfere with downstream amplification.

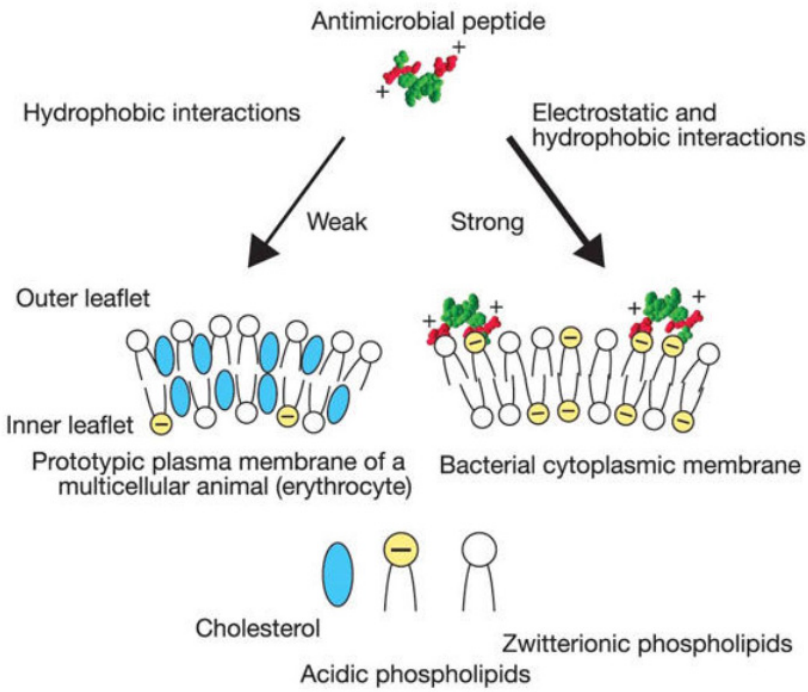

Figure 4. Basis of the AMP (antimicrobial peptide) membrane target specificity (based on (Zasloff, 2002, Matsuzaki, 1999). Basic (positively charged) amino acids of the AMP are shown in red and hydrophobic amino acids in green. The differences in bacterial and erythrocyte membrane structure that result in selectivity of the AMP interaction are shown.

1.6.1. Membrane-active AMPs: Cecropins, melittin, and magainins

Linear cationic α-helical peptides such as cecropins, magainins, and melittin act through disruption of the microbial cell.

Melittin, which has been isolated from honeybee venom, is a 26 amino acid long peptide with hydrophobic N-terminal region and predominantly hydrophilic C-terminal region (Kreil, 1973). Melittin severely disrupts lipid membranes, leading to cell lysis (Tosteson and Tosteson, 1981). In addition to wide scope antimicrobial properties, melittin also displays potent haemolytic activity (Conlon et al., 2003). Melittin is more active against Gram-positive bacteria, while its activity towards Gram-negative bacteria is highly sensitive to buffer ionic composition, probably due to the importance of the electrostatic interactions of peptide binding to LPS (Moerman et al., 2002).

Bombolitin is a component of bumblebee venom whose structure and activities are very similar to melittin. Bombolitins have a wide range of activity against both Gram-positive and -negative bacteria as well as pathogenic fungi in plants and erythrocytes (Argiolas and Pisano, 1985, Choo et al., 2010).

Magainins are a class of AMPs from the skin of the frog *Xenopus laevis* (Zasloff, 1987). Magainin analogues MSI-78 and MSI-594 are very potent AMPs that are active against numerous bacteria, including those resistant to conventional antibiotics (Maloy and Kari, 1995).

Cecropins are produced as an antibacterial response of the cecropia moth, with exception of cecropin P1, which was isolated from nematode within pig intestine (Hultmark et al., 1980, Andersson et al., 2003). Cecropins permealize cellular membranes to execute their antimicrobial activity towards fungi, viruses, Gram-positive bacteria (*Staphylococcus aureus, Bacillus subtilis*), and Gram-negative bacteria (*E. coli, Salmonella typhimurium, Serratia marcescens*) (Efimova et al., 2014, Guo et al., 2014).

To relieve the burden of the STI, POC-applicable, efficient, and accurate diagnostics are required. Current POC tests are mainly immunoassays, which lack the required sensitivity and specificity. The first steps for NAAT-based techniques application for POC diagnostics have been taken; however, no instrument-free solutions have yet been introduced, mainly because of the unresolved issue of nucleic acid preparation prior to amplification, which restricts easy application of NAAT in POC settings.

2. AIMS OF THIS STUDY

The main objective of the research described in this dissertation is to evaluate the feasibility and efficiency of isothermal nucleic acid amplification directly from clinical samples, with the focus on establishment of POC-applicable, fast, robust, and efficient technology including sample preparation prior to nucleic acid amplification. This includes evaluation of the analytical and clinical effect of direct nucleic acid amplification without prior template purification as well as establishment of the major factors affecting nucleic acid amplification directly from clinical urine samples.

- **Paper I**: The main aim of the paper was to establish an isothermal amplification-based *C. trachomatis* diagnostics assay for pathogen diagnostics directly from urine samples. We aimed to develop a recombinase polymerase isothermal amplification-based assay for application with minimally processed urine samples and to evaluate its sensitivity and specificity both clinically and analytically.

- **Paper II**: In this paper, we aimed to establish the major inhibiting components of urine samples affecting loop-mediated isothermal amplification. We aimed to evaluate the effect of DNA polymerase change on biological sample tolerance of the isothermal amplification.

- **Paper III**: The main aim of the paper was to establish the applicability and efficiency of the antimicrobial peptide-based sample pretreatment technique prior to isothermal amplification. We aimed to evaluate the efficiency of the different sample preparation techniques prior to amplification directly from urine samples spiked with *E. coli* bacteria.

3. METHODOLOGICAL CONSIDERATIONS

3.1. Selection of specific amplification target for *C. trachomatis*

Choosing a molecular target is a crucial step in amplification-based molecular diagnostics assay, determining both the sensitivity and specificity of the assay. The optimal nucleic acid target is highly conserved within different target pathogen serovars/strains but has sufficient variability with closely related species in particular. Multicopy gene targets are usually preferred as molecular diagnostics targets to obtain higher sensitivity of the assay.

For *C. trachomatis*-specific detection, we have selected a cryptic plasmid target. Several diagnostics assays for *C. trachomatis* detection target cryptic plasmid, as it has been established to be important to maintaining high virulence of the pathogen and is present in multiple copies per cell (Carlson et al., 2008, Seth-Smith et al., 2009, Pickett et al., 2005). For our amplification target, we have chosen a region within coding sequence 2 (CDS2) encoding pGP8 protein. RPA and LAMP primers were designed to recognize highly conserved region within CDS2 sequence (**Table 3**).

Gen-Bank sequence analysis with Basic Local Alignment Search Tool (BLAST) confirmed conservation of *C. trachomatis* amplification region among 11 *C. trachomatis* LGV biovar strains (serovars L1, L2, L2b, and L3; strains L1/224, L1/1322/p2, L1/115, L1/440/LN, L2/434/BU, L2b/UCH-1, L2b/UCH-2, L2b/LST, L2b/Ams1, L2b/Ams2, L3/404/LN) and 25 strains from a genital biovar (serovars D, E, F, G, Ia, J, and K; strains E-32931, E-547, E-160, E-103, E-DK-20, E/CS1025/11, E/SotonE8, E/SotonE4, E/Sweden3, E/Sweden2, E/Bour, E-8873, F-6068, F/CS847/08, F/Sweden5, F/Sweden4, Ia/SotonIa1, Ia/SotonIa3, K/SotonK1, J/UW-36, D/SotonD6, D/SotonD5, D-EC, D/UW-3, G/SotonG1). Oligonucleotides containing sequence elements that promote secondary structures and primer–primer interactions or hairpins were avoided. GAPDH housekeeping gene was used as an intrinsic control, as urine specimens contain up to 10^5/ml human epithelial cells (**Table 3**) (Morimoto et al., 2003).

3.2. Isothermal amplification and product detection

Two isothermal amplification methods, RPA and LAMP, have been used in this work.

The major benefits of RPA are its short amplification time compared to other isothermal amplifications and its tolerance for ambient operating temperatures. The optimal reaction temperature of the RPA (37–42˚C) is lower than for the majority of other isothermal amplifications, which makes it a good candidate for on-site applications. Furthermore, due to the lyophilized format, RPA reagents are highly stable at ambient temperatures.

RPA reaction was performed according to the manufacturer's (TwistDX) suggestion. Amplification was initiated through addition of magnesium acetate, incubated at 38°C, and terminated at 50°C where required. After four minutes of incubation, the reaction was mixed by flicking the tube. RPA primers were labelled with biotin and 6-carboxyfluorescein (FAM), enabling biotin-FAM double-labelled product detection on the PCRD-2 lateral-flow (LF) strip.

LF strip is a simple method for amplification product detection, allowing the result to be detected by the naked eye within a few minutes (**Figure 5**).

Table 3. RPA and LAMP amplification regions and primers used for specific *C. trachomatis* cryptic plasmid CDS2 sequence and *H. sapiens* GAPDH gene detection.

RPA *C. trachomatis* CDS2 **amplification product (5'–3') – 116 bp**		
CCTTCATTATGTCGGAGTCTGAGCACCCTAGGCGTTTGTACTCCGTCACAG CGGTTGCTCGAAGCACGTGCGGGGTTATTTTAAAAGGGATTGCAGCTTGT AGTCCTGCTTGAGAG		
Primer	5' modification	Sequence (5'–3')
FW	Biotin	CCTTCATTATGTCGGAGTCTGAGCACCCT AGGC
RV	FAM	CTCTCAAGCAGGACTACAAGCTGCAATC CCTT
RPA *H. sapiens* GAPDH **amplification product (5'–3') – 146 bp**		
AAGTCAGGTGGAGCGAGGCTAGCTGGCCCGATTTCTCCTCCGGGTGATGC TTTTCCTAGATTATTCTCTGGTAAATCAAAGAAGTGGGTTTATGGAGGTCC TCTTGTGTCCCCTCCCCGCAGAGGTGTGGTGGCTGTGGCATGGTG		
Primer	5' modification	Sequence (5'–3')
FW	Biotin	AAGTCAGGTGGAGCGAGGCTAGCTGGCC CGATT
RV	FAM	CACCATGCCACAGCCACCACACCTCTGC GGGGA
LAMP *C. trachomatis* CDS2 **amplification sequence (5'–3')**		
AATATCATCTTTGCGGTTGCGTGTCCTGTGACCTTCATTATGTCGGAGTCT GAGCACCCTAGGCGTTTGTACTCCGTCACAGCGGTTGCTCGAAGCACGTG CGGGGTTATTTTAAAAGGGATTGCAGCTTGTAGTCCTGCTTGAGAGAACG TGCGGGCGATTTGCCTTAACCCCACCATTTTTCCGGAGCGAGTTACGAAG ACAAAACCTCTTCGTTGACCGATGTACTCTTGTAGA		
Primer	5' modification	Sequence (5'–3')
F3	-	AATATCATCTTTGCGGTTGC
B3	-	TCTACAAGAGTACATCGGTCA
FIP	Biotin	TCGAGCAACCGCTGTGACGACCTTCATT ATGTCGGAGTC
BIP	FAM	GCAGCTTGTAGTCCTGCTTGAGTCTTCGT AACTCGCTCC
LF	Biotin	TACAAACGCCTAGGGTGC
LB	FAM	CGGGCGATTTGCCTTAAC

LAMP is known for its extremely high amplification efficiency and high specificity, as 4–6 primers used in a reaction recognize 6–8 distinct regions of the target gene. In addition to that, LAMP reaction is relatively low cost, which is an important benefit for diagnostics application.

LAMP reaction was performed as suggested by Eiken Chemical Co. Ltd (Notomi et al., 2000) at 63°C. Loop primers LF and LB were used in addition to B3, F3, FIP, and BIP primers to increase LAMP reaction speed and sensitivity. FIP and LF primers were labelled with biotin and BIP and LB primers with FAM so that amplification product could be detected by Amodia LF strips. Bsm DNA polymerase was generally used for LAMP amplification. When different DNA polymerases were used, LAMP reaction buffer pH, salt, Mg^{2+}, betaine, and dNTP concentrations were adjusted according to the manufacturer's suggestion. Detergents (Tween 20 or Triton X-100) were added to the reaction where required. For SD and OmniAmp DNA polymerases, LAMP reaction conditions were optimized experimentally (Jevtuševskaja et al., 2017). For quantitative LAMP (qLAMP), product formation was monitored in real time using Applied Biosystems 7900HT and EvaGreen fluorescent dye. ROX was used as a reference dye in the qLAMP reaction.

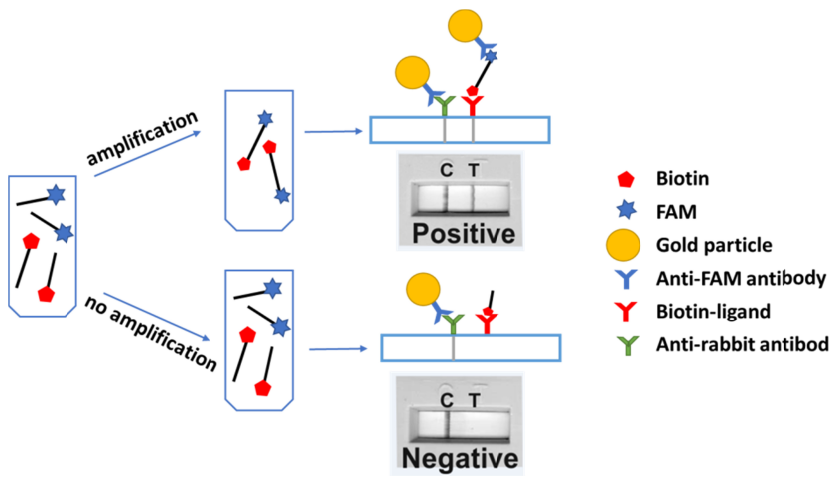

Figure 5. Schematic representation of lateral-flow strip-based amplification product detection.

3.3. Analytical sensitivity and specificity of the diagnostics assay

Molecular diagnostics assays are characterized using sensitivity and specificity values. Analytical sensitivity is the ability of the diagnostic test to detect target nucleic acid. Analytical sensitivity is usually expressed as the minimal detectable concentration of the target DNA (minimal template copy number per reaction required for amplification product formation), also referred to as limit

of detection (LOD). Analytical specificity refers to the ability of the assay to detect the particular target nucleic acid rather than others.

For diagnostic assay analytical sensitivity estimation, the following template DNA materials were used: (i) linearized pGL3-CDS2 plasmid (pGL3-Promoter vector carrying the *C. trachomatis* cryptic plasmid-encoded gene CDS2 (Krõlov et al., 2014)), (ii) *C. trachomatis* genomic DNA (gDNA) as total DNA isolated from HeLa-229 cells infected with *C. trachomatis* serovar J strain UW-36/Cx (ATCC® VR-886™), and (iii) human gDNA (RNA-free DNA purified from cultured HeLa cells).

Assay analytical specificity was estimated with 20–100 pg of gDNA from bacterial species potentially present in patient samples, such as *Mycoplasma genitalium* (ATCC 33530), *Neisseria gonorrhoeae* (ATCC-53422D), *Ureaplasma urealyticum* (NCTC 10177), and *E. coli* MG16.55 gDNA, as well as 1 ng of human gDNA.

3.4. Clinical sensitivity and specificity of the diagnostics assay

Clinical sensitivity refers to the test's ability to correctly detect patients who have the condition and shows the probability of a positive test given that the patient has the disease. Sensitivity is calculated as follows:

$$\text{Sensitivity} = TP/(TP + FN)$$ (**Table 4**)

Clinical specificity is the ability of the test to correctly identify patients who do not have the condition or the probability of a healthy person without the disease to be given a negative test result. Specificity is calculated as follows:

$$\text{Specificity} = TN/(TN + FP)$$

Table 4. Estimation of the clinical sensitivity and specificity of the diagnostic assay.

		Condition (determined by Cobas Amplicor *C. trachomatis* test)	
		Condition **positive**	Condition **negative**
Test outcome	**Positive**	**True positive** (TP)	**False positive** (FP)
	Negative	**False negative** (FN)	**True negative** (TN)

The sensitivity and specificity values alone can be misleading; therefore, they are often accompanied by a confidence interval (CI), which refers to the range of values within which the correct value lies at a given probability level (for example 95%).

For this work, clinical specificity, sensitivity, and 95% CIs were calculated using the clinical calculator from VassarStats (http://www.vassarstats.net/clin1.html). The reference method used for detection of *C. trachomatis* in clinical urine samples was a Roche Cobas Amplicor CT/NG test performed by the United Laboratories of Tartu University Hospital.

3.5. Collection and handling of clinical samples

Urine specimens were used for this work. Samples were collected from 70 patients (51 females and 19 males) attending a Sexual Health Clinic (Tartu, Estonia) during the period of April–May 2013. Approval for the study was obtained from the Research Ethics Committee of the University of Tartu. Patients were 18–25 years old, with a mean age of 21.5 years. Patient selection for STI analysis was performed by clinicians based on the following criteria: recent change of a sexual partner, multiple partners, unprotected sexual intercourse, STI detected in a partner, or STI symptoms (such as increased or abnormal vaginal discharge, abdominal pain, or painful urination). The first-void morning urine samples were self-collected. Mean sample volume was 25–35 ml (varying from 15 to 45 ml). Collected specimens were stored at 4°C and tested by RPA within one day of collection.

Simulated clinical samples were prepared by spiking pooled urine sample with *E. coli* DH5α-carrying pGL3-CDS2 plasmid. Bacteria was grown to mid logarithmic phase and washed with PBS prior to dilution in pooled urine to a concentration of $0.2–1\times10^5$ cells/µl.

Pooled patient urine samples (from five males and five females tested negatively for *C. trachomatis*) were used as a sample matrix to eliminate the aberrant effect due to naturally occurring high heterogeneity of the clinical samples.

3.6. Sample preparation prior to amplification

Several strategies were applied for sample pretreatment prior to amplification.

Total DNA was extracted from urine samples using the QIAamp Viral RNA Mini Kit (Qiagen). For heat treatment, samples were incubated 5 min at 90°C. For enzymatic lysis, samples were incubated for 5 min at RT°C with 1mg/ml of lysozyme in the presence of 1% Triton X-100 or 10 mM EDTA to enhance lysis efficiency for Gram-negative *E. coli*. For chemical lysis with mild detergent, samples were incubated for 5 min at RT°C in the presence of 1% Triton X-100. Alkaline lysis of the simulated urine samples was performed as a two-step

procedure, incubating samples for 5 min at RT°C with equal volume of lysis solution (400 mM KOH, 100 mM DTT, 10 mM EDTA) followed by neutralization with 400 mM HCl, 600 mM Tris HCl, pH 0.6.

The use of antimicrobial peptides (AMPs) for sample preparation was also studied. Samples were treated with selected membrane-active AMPs, like melittin, magainin analogue MSI-78 (pexiganan), againin-melittin MSI-594, bombolitin III, cecropin P1, and cecropin B analogue SB-37 (**Table 5**) for 5 min at RT°C with 50 µM final peptide concentration.

Table 5. List of the antimicrobial peptides used in the study and their sequences.

Peptide	Sequence	References
Melittin	GIGAVLKVLTTGLPALISWIKRKR QQ-NH_2	(Kreil, 1973)
MSI-78	GIGKFLKKAKKFGKAFVKILKK-NH_2	(Hallock et al., 2003)
MSI-594	GIGKFLKKAKKGIGAVLKVLTTGL -NH_2	(Porcelli et al., 2006)
Bombolitin III	IKIMDILAKLGKVLAHV-NH_2	(Argiolas and Pisano, 1985)
Cecropin P1	SWLSKTAKKLENSAKKRISEGIAIA IQGGPR	(Pillai et al., 2005)
SB-37	MPKWKVFKKIEKVGRNIRNGIVK AGPAIAVLGEAKALG	(Jaynes et al., 1989, Jaynes et al., 1988)

3.7. Determining main urine inhibitory components on LAMP

To evaluate the effect of urine on LAMP, several potential inhibitors were selected and tested at different concentrations. Urine samples have varying Na^+ ion concentrations, ranging on average from 50–190 mM. Therefore, LAMP efficiency was tested after addition of 5µl of 150 mM or 300 mM NaCl. Mg^{2+} ions have a direct effect on DNA polymerase activity and fidelity and therefore significantly affect amplification efficiency. Furthermore, Mg^{2+} ions affect annealing temperatures of several primer-template hybrids. Physiologically relevant Mg^{2+} concentration in urine ranges from 1.7–5.7 mM. We have tested LAMP activity in the presence of 0.1 mM, 0.5 mM, and 1 mM $MgSO_4$. Urea is known to prevent non-covalent bonding acting directly on the polymerase or hindering primer annealing (Hedman and Rådström, 2013). Normal concentration of urea in urine samples is 18 mg/l (Khan et al., 1991); we have tested the effect of addition of 1.5 mg/l, 15 mg/l, and 30 mg/l of urea on the LAMP reaction. The average concentration of albumin in urine is normally 10 mg/l but can be significantly increased in patients with conditions such as albuminuria. We have tested LAMP activity at BSA concentrations 0.07 mg/ml,

0.2 mg/ml, 0.7 mg/ml, 2 mg/ml, and 4 mg/ml. Normal urine can be slightly acidic or alkaline, with pH ranging on average from 4–9. Changes in pH can affect intermolecular bonds, and therefore enzymes are usually sensitive towards pH changes outside their optimal range. Therefore, addition of 5 µl of 100 µM HCl or 1 µM NaOH on LAMP efficiency was also tested.

3.8. *E. coli* membrane integrity and viability studies

To evaluate antimicrobial activity of selected AMPs, *E. coli* viability was assessed by colony-forming units (CFUs) count. *E. coli* cells were briefly treated with 50 µM AMP, and dilution series were plated for CFU count assessment. Viability was evaluated as a percentage of viable cells following AMP treatment as compared to untreated samples.

E. coli membrane integrity was also evaluated by flow cytometry (BD LSRII). The ability of propidium iodide to migrate into the *E. coli* cells was used to evaluate the cell lysis efficiency of different sample pretreatment techniques, such as heat, alkali, detergent, lysozyme, and treatments with different AMPs.

4. RESULTS AND DISCUSSION

The research work described in this thesis addresses the issue of implementation of nucleic acid amplification-based diagnostics in POC applications. Although NAAT tests offer high sensitivity and specificity levels, they are difficult to implement in an instrument-free way. Additionally, the nucleic acid purification requirement prior to amplification is considered a major obstacle for NAAT POC application.

In the first paper, we used isothermal amplification for *C. trachomatis* specific diagnostics from heat-treated patient urine samples. Isothermal amplification enables instrument-free detection of target nucleic acids and is considerably less sensitive towards biological sample material, allowing direct amplification from urine samples. In the second paper, we address the issue of urine sample inhibition on loop-mediated isothermal amplification, determine the major inhibiting components, and propose methods for overcoming the inhibition. The third paper addresses the requirement and efficiency of sample pretreatment prior to amplification. Here we also introduce a novel sample pretreatment method using antimicrobial peptides and compare efficiency of different sample pretreatments using simulated samples (*E. coli*-spiked urine samples).

4.1. Sensitive and rapid detection of *Chlamydia trachomatis* directly from urine samples (Paper I)

Nucleic acid purification prior to amplification significantly affects the cost and complexity of the diagnostics assay. Furthermore, the procedure is poorly applicable in POC settings. In this paper, we have developed a *C. trachomatis* diagnostics assay that allows fast and sensitive detection of pathogen directly from urine samples, therefore allowing forgoing of the nucleic acid purification step.

For specific and sensitive *C. trachomatis* detection, we have chosen a new amplification target, a CDS2 region located on the cryptic plasmid. This target region is highly conserved among different *C. trachomatis* serovars and is present in multiple copies per cell. The *C. trachomatis* diagnostic target was chosen so it would also cover the Swedish plasmid variant-carrying strains (Seth-Smith et al., 2009). As clinical samples usually contain patient cellular material, a human DNA target within the GAPDH gene was chosen for the intrinsic control of the reaction. Intrinsic reaction control is required for distinguishing negative test results from invalid reactions.

A *C. trachomatis*-specific target was amplified using RPA isothermal amplification. For fast and sensitive detection of the amplification, LF strips were used, which allowed naked-eye end-point result visualization within minutes. RPA proved to be highly sensitive, detecting as low as 50 target DNA

copies per reaction, which translates to 5–12 pathogen cells per analysis (depending on the plasmid copy number).

3–12 min minimal reaction times have been reported for RPA depending on the template concentration (Wang et al., 2017). We have determined that in our settings, RPA reaction time could be reduced to 10 min without affecting the detection limit. This correlates well with previous findings that 10–12 min is the required time to result in RPA reactions at template concentrations near LOD. RPA reaction time can be reduced even to 3 min when high template DNA concentrations are used (Wang et al., 2017).

We have also established that RPA can tolerate up to 10% urine in the reaction without significant loss of assay analytical sensitivity. Although whole-blood has been shown to be quite inhibiting to the RPA reaction (Kersting et al., 2014), our findings show RPA to be relatively tolerant towards urine specimens. Amplification was possible even when urine concentration was increased to 20% in the RPA reaction; however, that high sample concentration was affecting the signal intensity on the LF strips. Thus, urine specimens are significantly less inhibiting to RPA reaction than to PCR (Khan et al., 1991), and RPA can potentially be applied directly to urine samples without prior nucleic acid purification.

To our knowledge, this was the first work describing the effect of urine on RPA assay and attempting amplification directly from minimally processed clinical samples. RPA amplification from urine samples has been further confirmed by other researchers (Valiadi et al., 2016, Rosser et al., 2015). However, 10% urine was found inhibiting by (Rosser et al., 2015) at the near LOD template concentration. This might be explained by high heterogeneity of urine samples, with some specimens being more inhibiting to amplification than others. We have addressed this issue by using pooled specimens from ten different patients to reduce the aberrant effect due to sample heterogenicity.

To confirm our analytical findings, we have performed a small-scale clinical study to evaluate RPA-based *C. trachomatis* detection efficiency directly from urine samples of 70 patients. Heat treatment has been shown to be a relatively efficient sample pretreatment technique prior to isothermal amplification directly from several biological samples (Nyan et al., 2014, Hill et al., 2008, Sun et al., 2014, Jain et al., 2011). We have heated patient urine samples for 5 min at 90°C prior to RPA amplification. Clinical evaluation of RPA-based assay combined with heat treatment resulted in 83% sensitivity (95% CI, 51%–97%) and 100% specificity (95% CI, 92%–100%). Obtained clinical sensitivity values are significantly higher than the average 40–60% sensitivity reported for *C. trachomatis* POC immunoassays (Yin et al., 2006, Bandea et al., 2009, Sabidó et al., 2009, van der Helm et al., 2012). However, nucleic acid purification prior to RPA analysis increased clinical sensitivity values to 100% (95% CI, 70%–100%). Therefore, improvement of direct nucleic acid amplification sensitivity should be addressed by future research.

We and others have shown direct amplification from urine samples with other isothermal amplification methods such as LAMP (Nakano et al., 2015,

Hill et al., 2008, Jevtuševskaja et al., 2016). Nakano et al. have achieved comparable clinical sensitivity with LAMP directly from unprocessed samples as compared to purified DNA samples. In our study, clinical sensitivity was reduced from 100% to 83% when heat-treated urine samples were used instead of purified DNA. Only 55% clinical sensitivity was achieved for *C. trachomatis* detection with LAMP from unprocessed samples, and 64% sensitivity for heat-treated samples (Jevtuševskaja et al., 2017). The difference in clinical performance is probably due to the different amplification methods, different target pathogens, and use of clinical versus simulated samples. The possibility for direct amplification from minimally processed clinical samples exists, although its reliability needs further clinical confirmation. Further studies should also address the need and efficiency of sample processing prior to isothermal amplification.

In addition, our clinical study has confirmed previous findings concerning the lack of correlation between symptoms and *C. trachomatis* infection. Only 25% of *C. trachomatis*-positive patients were symptomatic, while 26% of *C. trachomatis* negative patients also displayed symptoms that could be associated with the STI, emphasizing again the need for routine screening in order to prevent further spread of the STI.

The major advantage of the developed diagnostics assay is its speed, as the whole process, including sample processing, can be completed within 15–20 min (**Figure 6**). Up to 30 min performance time is generally accepted for on-site POC diagnostics tests. The developed *C. trachomatis* RPA assay meets this requirement. In addition to being an extremely fast amplification technique, RPA also tolerates ambient incubation temperatures and uses lyophilized reagents, making it a good candidate for POC applications. With LF naked-eye result readout, RPA carries the potential to be applied in a completely instrument-free manner.

However, the biggest advantage of the developed *C. trachomatis* assay is its high specificity and relatively high sensitivity levels. The specificity of 100% (95% CI, 70%–100%) greatly surpasses Gram staining and urine flow cytometry, and sensitivity of 83% (95% CI, 51%–97%) is far above the average immunoassay sensitivity, including those of Quickview, ACON, Biorad, or Diagnostics for the Real World rapid *C. trachomatis* tests. The sensitivity of the isothermal amplification-based POC assay can be further improved by reducing the inhibitory effect of the biological samples and through optimization of the sample pretreatment procedure. Both of these aspects will be addressed further in Papers II and III presented in this dissertation.

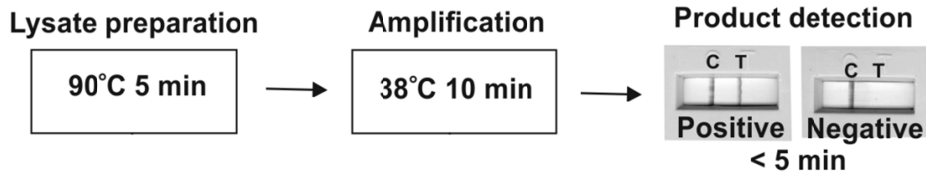

Figure 6. RPA-based *C. trachomatis* detection assay layout from sample to result. Urine samples were prepared for amplification by simple heating procedure. Recombinase polymerase amplification (RPA) was then performed directly from urine lysates. FAM-biotin double labelled amplification product was then detected using lateral-flow technology.

4.2. Tolerance of urine and its major inhibitors by different DNA polymerases in loop-mediated isothermal amplification (Paper II)

The inhibiting effect of biological sample components on nucleic acid amplification is a significant concern for amplification from crude biological samples in particular, but also because traces of inhibiting components can be present in purified nucleic acid samples. This can lead to reduced sensitivity of the assay by causing a false negative effect. We have addressed this issue by analyzing the effect of the urine samples and their components on LAMP isothermal amplification.

There are indications that amplification inhibitors majorly affect DNA polymerase activity, meaning that change of the polymerase can have a significant effect on the assay inhibitor tolerance (Baar et al., 2011, Ghadessy et al., 2001, Kermekchiev et al., 2009). We have selected 11 DNA polymerases (Bsm, Bst, Bst2.0, Bst2.0 WarmStart, GspSSD, GspSSD2.0, GspM, GspM2.0, Tin, OmniAmp, and SD DNA polymerase) and analyzed their speed and urine tolerance in LAMP reaction.

GspSSD2.0 polymerase was significantly faster than others with an estimated time to result of only 10 min, while Tin and SD DNA polymerases were slowest, requiring at least 35 min. Other tested polymerases had similar speed ranges, achieving results in 17–25 min.

Bsm polymerase displayed the highest tolerance towards urine, with no change in reaction speed even in the presence of 20% urine. Bst2.0, GspM2.0, and GspSSD2.0 were also able to tolerate moderately the presence of 10% urine in the reaction, producing results within 20 min. SD, OmniAmp, and Tin polymerases had the lowest tolerance towards clinical samples, and therefore should not be the primary choice for direct amplification. Notably, SD polymerase is a Taq-based mutant carrying additional strand-displacement activity. Taq polymerase is known to have high sensitivity towards biological samples in PCR reaction (Khan et al., 1991, Al-Soud and Rådström, 2001, Abu Al-Soud and Rådström, 2000); therefore, these results for SD polymerase could be have

been expected. In addition, our results confirmed that biological sample inhibition is majorly due to the effect on DNA polymerase activity.

To investigate the nature of urine inhibition, we next tested LAMP amplification in the presence of several potential inhibitory components. Major known amplification inhibitors of urine samples are urea and high salt (K^+, Na^+, Mg^{2+}) concentrations (Schrader et al., 2012). Urine is also known to have high protein content under certain conditions; thus, we have tested the effect of addition of the BSA to LAMP. Due to their highly heterogeneous nature, urine samples' pH can vary from 4–9; therefore, we have also addressed this effect on LAMP.

Addition of urea, BSA, $MgSO_4$, alkali, or acid to the reaction (at tested concentrations) had no significant effect on LAMP amplification efficiency for any of the tested polymerases. Confirming previous findings, LAMP reaction proved to be significantly less sensitive towards urea addition than PCR (Khan et al., 1991, Edwards et al., 2014). Addition of NaCl to the reaction, on the other hand, had a major effect on LAMP reaction that correlated well with urine intolerance. Polymerases like OmniAmp, Tin, and SD were highly sensitive towards NaCl and urine addition to the reaction. Amplification efficiency of the Bsm polymerase was not affected by urine addition, nor was reaction speed reduced upon increased NaCl concentration.

These findings indicate that high salt concentration in urine could be the main inhibiting component of LAMP. High salt concentration could act by influencing binding of the DNA polymerase to the template. Thus, DNA polymerases with stronger affinity towards the template could be less affected by salt addition and could possibly tolerate higher quantities of urine sample.

It should be noted that GspM2.0 and GspSSD2.0 polymerases were quite sensitive towards urine addition, although their reaction speed was not affected by NaCl. Therefore, clinical sample inhibition of these polymerases is probably implemented by a different effector.

In conclusion, our research showed that LAMP isothermal amplification is less sensitive towards urine sample addition than PCR, which is in accordance with previous findings and confirms the possibility of isothermal NAAT application for direct amplification from clinical samples. The selection of polymerase used in NAAT significantly affects speed and biological sample tolerance. Thus, individual amplification reaction optimization is required to achieve optimal results. Our findings show that GspSSD2.0 polymerase should be selected when the fastest time to result is required. Bsm polymerase displayed high tolerance towards urine sample addition to the reaction. Additionally, high Na^+ concentration was found to be majorly responsible for LAMP inhibition; thus, omission of salt from the reaction buffer could be considered for direct amplification from urine samples.

4.3. Implementation of membrane-active antimicrobial peptides for sample preparation prior to isothermal nucleic acid amplification (Paper III)

The finding that isothermal amplification techniques could tolerate the presence of biological samples in the reaction created the opportunity for direct amplification from minimally processed samples, allowing forgoing of the nucleic acid purification step. Although unprocessed samples can sometimes be directly applied for amplification (Hoos et al., 2017, Hill et al., 2008, Nakano et al., 2015), a simple sample preparation procedure is generally required for increased sensitivity of the assay (Sun et al., 2014, Nie et al., 2012, Wang et al., 2016, Kongkasuriyachai et al., 2017).

Heat, detergent, alkali, and enzymatic treatments have been applied prior to LAMP amplification (Nakanishi et al., 2011, Sun et al., 2014, Wang et al., 2016). Several of these well-established sample pretreatment techniques have limitations such as low efficiency, multi-step procedure, sensitivity towards buffer components, instrument requirements, and inapplicability to some pathogen types. In this work, we have investigated the possibility of urine sample pretreatment with antimicrobial peptides (AMPs) prior to amplification and compared it to other well-established sample processing techniques.

AMPs often act through disruption of cellular membrane on wide spectra of microbes such as bacteria, viruses, fungi, and protozoa. In the context of diagnostics, lysis of cells is important for release of the nucleic acids that are used as a target material for amplification. We have shown that AMPs are highly efficient in bacterial cell disruption in the biological sample environment, lysing the cells completely within 5 min incubation at RT°C. Heat and alkali treatment resulted in comparable cell lysis to that of AMP treatment, while detergent and lysozyme treatment were less effective on *E. coli*.

Inhibitors of the amplification reaction are a major concern with unpurified samples. In addition to the inhibitors present in the biological sample, sample pretreatment materials can also be inhibiting to the reaction. We have established that LAMP isothermal amplification is particularly sensitive towards addition of alkaline lysis components and Triton X-100. Several AMPs can bind nucleic acids in a non-specific manner due to the positive charge of the peptides. Magainin analogues (MSI-78 and MSI-594) were highly inhibiting to the reaction, while cecropin family peptides (Cecropin P1 and SB-37) had a moderate effect on LAMP amplification.

To investigate the efficiency of different sample pretreatment techniques prior to LAMP amplification, we have spiked urine samples with *E. coli* bacteria. Our results showed that pretreatment of the sample was important for optimal amplification sensitivity. In our hands, Cecropin P1 pretreatment of simulated urine samples was most efficient, followed by heat treatment. Alkali and SB-37 pretreatment also resulted in moderate increases in the amplification template amount. These results correlate with clinical results of *C. trachomatis*

detection by LAMP amplification from unprocessed, heat-treated, and AMP-treated urine samples (respective clinical sensitivity values are 55%, 64%, and 73%) (Jevtuševskaja et al., 2016).

These findings show the potential applicability of AMPs for biological sample pretreatment prior to isothermal amplification. Major advantages of AMP pretreatment over other conventional methods are that it is a fast, room-temperature applicable, one-step procedure with potential applicability for a wide spectrum of microbes. Therefore, AMP pretreatment could be considered a viable candidate for POC applications.

In general, our work has shown that sample pretreatment is required prior to NAAT, as it helps to release intracellularly located template nucleic acids. Efficient sample pretreatment can have a significant impact on clinical and analytical assay sensitivity. Inhibition of sample pretreatment components on downstream amplification can be a concern. Further research should address AMP pretreatment applicability with different clinical sample materials and target organism types.

5. CONCLUSIONS

This work evaluated a possible use of nucleic acid amplification directly from urine samples without prior purification of target DNA. Direct nucleic acid amplification was evaluated analytically using two isothermal amplification techniques: loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA). Different sample preparation methods were applied for sample pretreatment prior to amplification with AMP treatment to give the best results. Potential biological sample inhibitors were determined, and isothermal amplification assay condition adjusted accordingly to allow direct amplification from clinical samples. Direct amplification was also evaluated with a small-scale clinical study. Results of this work are particularly valuable for pathogen diagnostics in POC applications.

The key findings from the research presented in this dissertation are listed below:

- **Paper I.** In this study, we developed a POC-applicable *C. trachomatis* detection assay. We chose RPA isothermal amplification for sensitive and specific pathogen detection directly from heat-treated urine samples. The developed assay could detect as few as 5 pathogens per reaction and had clinical sensitivity and specificity of 83% and 100%, respectively. The whole procedure was fairly simple and did not require specific laboratory instrumentation or trained personnel, and the reaction result could be visualized within 20 min using lateral-flow dipstick technology.
- **Paper II.** Here we evaluated the efficiency of several DNA polymerases for amplification directly from urine samples in LAMP isothermal amplification. We established the amplification speed of 11 commercially available polymerases and their tolerance towards urine samples and major inhibitory components. We found that the choice of polymerase is crucial for optimal speed and sensitivity of isothermal amplification directly from clinical samples. We also identified high salt as a major inhibitor present in the urine samples affecting LAMP amplification efficiency.
- **Paper III.** In this study, we applied antimicrobial peptides for urine sample pretreatment prior to isothermal amplification. Using urine samples spiked with *E. coli* bacteria, we established that pretreatment with cecropin P1 AMP increased amplification target DNA to six times that of untreated urine on average. We have also evaluated heat, alkali, enzymatic, and detergent treatment efficiency for urine sample pretreatment prior to isothermal amplification and found that AMP treatment was most efficient, followed by heat treatment.

SUMMARY IN ESTONIAN

Nukleiinhapete amplifikatsioonil põhinev diagnostika otse kliinilistest proovidest *Chlamydia trachomatis* detektsiooni näitel uriini proovist

Seksuaalsel teel levivad infektsioonid (STLI) on oluliseks koormaks tervishoiusüsteemile. Iga päev nakatub nendesse üle miljoni inimese maailmas. STLI-de diagnoosimine on keeruline, sest enamus haigusi ei põhjusta mingeid sümptomeid. Samas ravimata jäänud suguhaigused võivad põhjustada kroonilisi põletikulisi reaktsioone ning avaldada selle kaudu olulist mõju inimese reproduktiivsele tervisele. Suureks probleemiks on ka infektsioonide ülekandumine sünnitavalt emalt lapsele ning STLI-de põhjustatud tüsistused raseduse ajal.

Kõrgesse riskigruppi kuuluvate inimeste ja rasedate skriinimine on ennast tõestanud efektiivne strateegia STLI-de leviku piiramiseks ning nendega seonduvate tüsistuste vältimiseks. Rutiinne STLI diagnostika vajab aga usaldusväärseid ja kiireid lahendusi, mille puudumist peetakse peamiseks STLI-de kontrollimatu leviku põhjuseks. Tsentraalsetesse laboritesse koondunud diagnostika meetodid on suures osas automatiseeritud ning pakkuvad väga täpseid tulemusi. Kuid nende hind ja infrastruktuuri vajadus piirab laiahaardelist rakendamist. Üldlevinud arvamuse kohaselt aitab STLI-de levikut piirata ennekõike usaldusväärse kiirtesti olemasolu, mis võimaldab haigustekitajat diagnoosida arstivisiidi käigus.

Chlamydia trachiomatis-t võib pidada levinuimaks suguhaiguste põhjustajaks. Igal aastal nakatub *C. trachomatis-ega* ligi 130 miljonit inimest üle maailma, ning selle esinemissagedus seksuaalselt aktiivsete noorte seas võib ulatuda kuni 20%-ni. Praegu turul olevad *C. trachomatise* kiirtestid põhinevad immunoanalüüsil ning on üsna vähe tundlikud. Piisava tundlikkuse puudumine piirab nende laialdasemat kasutuselevõttu.

Antud doktoritöös esitatud teadustulemused on suunatud nukleiinhapete (DNA) amplifikatsioonil põhineva diagnostika rakendamisele kiirdiagnostikas. Kiirdiagnostika vajab ennekõike kiireid (soovitavalt alla poole tunniseid) meetodeid, mis võimaldavad väga täpselt ennustada patogeeni olemasolu proovis, soovitavalt täiesti instrumendivabalt. Klassikaline DNA amplifikatsiooni meetod PCR vajab temperatuuri tsüklilist muutumist, mistõttu ei sobi hästi instrumendivabaks rakenduseks. Selles töös töötati välja isotermilisel amplifikatsioonil põhinevat *C. trachomatise* diagnostika meetod, mille suurimaks eeliseks on, et see on teostatav kasutades ainult termostaati. Väljatöötatud meetodis piisab 10 minutit kestvast amplifikatsiooni reaktsioonist *C. trachomatise* DNA olemasolu tuvastamiseks kliinilisest proovist. Amplifikatsiooni produkti olemasolu on palja silmaga registreeritav kasutades külgvoolu immunokromatograafilist detektsiooni süsteemi, mis võimaldab kohest kvalitatiivse vastuse saamist. Võrdluseks tasub mainida, et enamus laboridiagnostika meetodeid

vajab vastuse saamiseks aega mitu tundi. Väljatöötatud meetodi kliiniline analüüs näitas selle sensitiivsuseks ja spetsiifilisuseks 100%.

Lisaks potentsiaalsele instrumendivabale rakendusele on isotermilise amplifikatsiooni suurimaks plussiks suurem tolerantsus bioloogiliste proovide materjali suhtes. Meie juhul võimaldas see omadus viia läbi *C. trachomatise* DNA amplifikatsiooni otse uriini proovidest ilma eelneva nukleiinhapete puhastamiseta. Eelnevalt kuumutatud uriiniproovide kasutamine otse amplifikatsiooniks tagas kliinilises uuringus 83% sensitiivsuse. See on oluliselt suurem teiste kiirmeetoditega võrreldes, mille kliiniline sensitiivsus jääb keskmiselt 40% juurde.

Võimalus vältida nukleiinhapete puhastamist enne amplifikatsiooni on väga suureks eeliseks kiirdiagnostika rakenduste jaoks, tehes metoodika mitte ainult odavamaks, vaid ka oluliselt lihtsamaks. Samas tõstatab DNA amplifikatsioon otse kliinilistest proovidest küsimusi, mis puudutavad nii bioloogiliste proovimaterjalide inhibitsiooni amplifikatsioonireaktsioonile kui ka eeltöötluse vajaduse nukleiinhapete vabastamiseks rakkude seest.

Meie näitasime, et isotermilised amplifikatsioonimeetodid on oluliselt tolerantsemad kliinilise proovimaterjali lisamise suhtes reaktsioonile kui PCR. Nii näiteks suudab isotermiline LAMP amplifikatsioon (loop-mediated isothermal amplification) töötada kuni 20% uriini juuresolekul, ilma et reaktsiooni kiirus oleks mõjutatud. Samuti selgus meie uuringutest, et peamiseks amplifikatsiooni reaktsiooni inhibiitoriks on uriini proovides sisalduv kõrge sool (Na^+ ioonide näol). Inhibiitorite mõju avaldub peamiselt DNA polümeraasi aktiivsuse inhibeerimise kaudu. Selle tõttu on üheks toimivaks mehhanismiks diagnostika meetodi parandamiseks tolerantse DNA polümeraasi selektsioon või geneetiline modifikatsioon, mis selgus ka meie katsetes.

Et suures osas asub sihtmärk DNA rakkude sees, siis üldjuhul vajavad kliinilised proovid töötlemist, mis võimaldaks DNA vabastamist rakkudest. Me võrdlesime erinevate proovi eeltöötlemise meetodite efektiivsust nagu kuumutamine, aluseline töötlus ning töötlus detergendi või lüsotsüümiga. Uue strateegiana rakendasime membraan-aktiivseid antimikroobseid peptiide proovimaatriksis sisalduvate patogeeni rakkude lüüsiks. Meie tulemused näitasid, et proovi eeltöötlus on vajalik ning võimaldab oluliselt tõsta amplifikatsiooni efektiivsust. Samuti näitasime, et antimikroobsete peptiididega töötlus on võrreldud meetoditest kõige efektiivsem.

Kokkuvõtteks näitas uurimistöö amplifikatsiooni võimalikkust otse bioloogilisest proovist ilma eelneva nukleiinhapete puhastamiseta adresseerides ka bioloogilise proovi inhibitsiooni ja eeltöötluse teemasid.

ACKNOWLEDGEMENTS

The research presented in this thesis was carried out in the Institute of Technology, University of Tartu, and supported by grant EU32415 from Enterprise Estonia (European Regional Development Fund) and Sächsische AufbauBank grant SAB#100204668, as well as grants from Estonian Ministry of Education and Research (SF0140031Bs09, SF0180027s08, IUT20-26, PUT753) and European Regional Development Fund through the projects Center of Excellence in Molecular Cell Engineering (2014-2020.4.01.15-0013).

First and foremost, I would like to express my deep gratitude to my supervisors Prof. Ülo Langel and Dr. Indrek Tulp. I really appreciate Prof. Ülo Langel's support and encouragement that led to the completion of this thesis, and I am deeply grateful for this opportunity. I would like to thank Indrek for introducing me to the inspiring field of molecular diagnostics, for always positive and friendly attitude, for interesting discussions and scientific advice. I would also like to thank Marko Lehes for motivation and keeping the team spirit.

I would like to acknowledge and thank my current and past coworkers and coauthors of the publications: Julia Uusna, Tiia Grellier, Piret Arukuusk, Ly Pärnaste, Kristina Kiisholts, Kadri Künnapuu, Krista Freimann, and others. I especially appreciate the support and help of Jekaterina Jevtuševskaja throughout our long-term collaboration and mutual research efforts. I am also very thankful to Kaido Kurikoff for the valuable advice. It has been a great pleasure to be a part of the Molecular Biotechnology group throughout these years.

I really appreciate the support and help of my friends and family. I would like to particularly thank Anu, for inspiration, support, appreciation of my achievements and so much more. I am deeply grateful to Ahto for his constant support, valuable advice and for always being there for me. And finally, I would like to dedicate this work to my girls and express my gratitude towards the love and joy they bring into my life.

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PUBLICATIONS

CURRICULUM VITAE

Name: Katrin Krõlov (formerly Viikov)
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Careers and positions

2014–…	Research fellow/project consultant Selfdiagnostics OÜ
2012–2013	Research fellow/project manager Selfdiagnostics OÜ
2012–2013	Research Fellow in molecular biomedicine University of Tartu, Faculty of Science and Technology, Institute of Technology, University of Tartu
2011–2012	Research Fellow in General and Microbial Biochemistry University of Tartu, Faculty of Science and Technology, Institute of Molecular and Cell Biology, University of Tartu
2008–2009	Research Fellow Smurfit Chair of Medical Genetics, Smurfit Institute of Genetics, Trinity College Dublin, Ireland.

Education

2010–…	MBA studies in entrepreneurship and technology management, University of Tartu
2006	MSc degree in transgenic technology, University of Tartu
2004	BSc degree in transgenic technology, University of Tartu
2000	Kohtla-Järve Järve High school diploma

Research related administrative and managerial work

2010–2011	supervision of the BSc thesis of Kaarel Kruuse "Membrane association of the *Saccharomyces cerevisiae* mitochondrial DNA polymerase"
June 2010	organisation of the international Summer school in Molecular Biology „Mitochondria, metabolism and homeostasis", Palmse manner, Harjumaa
Mai 2008	organisation of the mini conference in biochemistry "Protein complexes formation and biosynthesis of the macromolecules", Greete motel, Valgamaa

Science awards and recognitions

2010	Award for Best Poster at the "Evolving DNA polymerases" conference, Switzerland

Publications

Krõlov, K., Uusna, J., Anmann, T., Andresen, L., Jevtuševskaja, J., Tulp, I., and Langel, Ü. (2017) Implementation of antimicrobial peptides for sample preparation prior to nucleic acid amplification in point-of-care settings. Expert Rev Mol Diagn. 2017 Oct 9:1–9

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Industrial property

Indrek Tulp, Katrin Krõlov, Marko Lehes, Ülo Langel, “Method and its compositions for detection of nucleic acid target from biological samples and body fluids” (Priority date 20.10.2012), WO2014060604, AU2013333763, CA2888949, EP2922966, JP2015537284, US20150322493.

Katrin Krõlov, Ülo Langel, Indrek Tulp, “Method for the detection of a sexually transmitted infectious pathogen” (Priority date 18.12.2015), WO2017103269.

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Töökohad ja ametid

2014–…	Teadur/projekti konsultant Selfdiagnostics OÜ
2012–2013	Teadur /projekti juht Selfdiagnostics OÜ
2012–2013	Molekulaarse biomeditsiini teadur Tartu Ülikool, Loodus- ja tehnoloogiateaduskond, Tehnoloogiainstituut
2011–2012	Üldise- ja mikroobibiokeemia teadur Tartu Ülikool, Loodus- ja tehnoloogiateaduskond, Molekulaar- ja Rakubioloogia instituut
2008–2009	Teadur Smurfit Chair of Medical Genetics, Smurfit Institute of Genetics, Trinity College Dublin, Ireland.

Haridustee

2010–…	MBA õpe ettevõtlus ja tehnoloogia juhtimise erialal, Tartu Ülikool
2006	MSc kraad transgeenses tehnoloogia, Tartu Ülikool
2004	BSc kraad transgeenses tehnoloogia, Tartu Ülikool
2000	Kohtla-Järve Järve Gümnaasiumi tunnistus

Teadusorganisatsiooniline ja -administratiivne tegevus

2010–2011	Juhendamine, Kaarel Kruuse bakalaureuse töö “Saccharomyces cerevisiae mitokondriaalse DNA polümeraasi assotsiatsioon membraanidega”
Juuni 2010	rahvusvahelise molekulaarbioloogia alase suvekooli korraldamine “Mitochondria, metabolism and homeostasis”, Palmse mõis, Harjumaa
Mai 2008	biokeemia kevadkooli “Valgukomplekside moodustumise ja makromolekulide biosünteesi mehhanismid” korraldamine, Greete motell, Valgamaa

Teaduspreemiad ja tunnistused

2010	Parima poster-ettekande auhind rahvusvahelisel konverentsil “Evolving DNA polymerases”, Šveits

Publikatsioonid

Krõlov, K., Uusna, J., Anmann, T., Andresen, L., Jevtuševskaja, J., Tulp, I., and Langel, Ü. (2017) Implementation of antimicrobial peptides for sample preparation prior to nucleic acid amplification in point-of-care settings. Expert Rev Mol Diagn. 2017 Oct 9:1–9

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