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Supervisors: Prof. Ülo Langel,
Institute of Technology, University of Tartu, Estonia
Department of Neurochemistry, Stockholm University, Stockholm, Sweden

PhD Julia Uusna
Institute of Technology, University of Tartu, Estonia

Opponent: Dr. Dirk Kuhlmeier,
Fraunhofer Institute for Cell Therapy and Immunology

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ABSTRACT

More than 30 different bacteria, viruses, and parasites can be transmitted through sexual contact and cause sexually transmitted diseases. Although common symptoms of these infections include vaginal discharge, penile discharge, ulcers on or around the genitals, and pelvic pain, they often result in minimal or no symptoms which increases the risk of passing the disease on to others during sexual contact. If left untreated, such infections can often lead to major health concerns, such as ectopic pregnancy, female infertility, or chronic pelvic pain. As such, prompt and accurate identification of pathogens and early stage detection of infections helps to prevent the development of long-term complications and reduce the risk of co-infections.

Much work has been devoted to developing rapid, on-site, sensitive, specific, reliable, and cost-efficient pathogen detection methods for large-scale screening in low and middle-income countries. Currently, most reference methods for the detection and identification of pathogens rely on culture or PCR-based techniques that can be both sensitive and specific, yet, are often time-consuming and labor intensive because they require extensive sample preparation, expensive laboratory apparatus, and trained personnel. This effectively limits their usage to clinical facilities. Therefore, there is a pressing need to develop affordable, accessible, and effective techniques that can form the basis for a point-of-care diagnostics platform that can find application in low resource settings with limited or no access to laboratory services.

Isothermal nucleic acid amplification assays provide an effective alternative to culture- or PCR-based diagnostics and do not require a thermocycling machine. These methods provide accurate and reliable results with reduced time and higher throughput and are more tolerant to inhibitory components found in biological samples, which simplifies their applicability and integration into point-of-care diagnostic workflows.

This work presents an efficient application of two rapid and sensitive isothermal amplification methods – Loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA) to detect the sexually transmitted pathogen *Chlamydia trachomatis* (CT) directly from urine samples without prior DNA purification. We also compared commercially available polymerases that possess strand displacement activity to both assess their tolerance to inhibitors within the biological samples and provide a means to estimate which polymerase most effectively amplifies genomic DNA directly within crude samples. This approach reduces both the analysis time and the final cost of the diagnostic tool. These amplification techniques can form the basis for developing sensitive, robust, and inexpensive one-step isothermal amplification based molecular assays for point-of-care screening of pathogens directly from biological samples.

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications which 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. doi: 10.1016/j.jmoldx.2013.08.003.

II. **Jevtuševskaja, J.,** Uusna, J., Andresen, L., Krõlov, K., Laanpere, M., Grellier, T., Tulp, I., and 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. doi: 10.1186/s12879-016-1674-0.

III. **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 Jan 29:1–8. doi: 10.1080/14737159.2017.1283218.

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My personal contribution to each article referred to in this thesis is as follows:

I. performed the experiments and participated in the data analysis.

II. designed and performed many of the experiments, analyzed most of the data and participated in the writing of the manuscript as a corresponding author;

III. designed and performed most of experiments, analyzed all the data and wrote the manuscript as a corresponding author.

Other publications:

IV. Kõiv, V.; Andresen, L.; Broberg, M.; **Frolova, J.;** Somervuo, P.; Auvinen, P.; Pirhonen, M.; Tenson, T.; Mäe, A. (2013). Lack of RsmA-Mediated Control Results in Constant Hypervirulence, Cell Elongation, and Hyperflagellation in Pectobacterium wasabiae. PLoS ONE, 8 (1), e54248, journal.pone.0054248.

V. Andresen, L.; **Frolova, J.;** Põllumaa, L.; Mäe, A. (2015). Dual role of RsmA in the coordinated regulation of expression of virulence genes in Pecto-bacterium wasabiae strain SCC3193. Microbiology-SGM, 161 (11), 2053−2264, 10.1099/mic.0.000159

ABBREVIATIONS

CFT	Complement fixation tests
CT	Chlamydia trachomatis
DFA	Direct fluorescent antibody
EB	Elementary body
EEA	European Economic Area
EIA	Enzyme immunoassay
EU	European Union
FAM	Fluorescein amidite
HDA	Helicase dependent amplification
LAMP	Loop mediated isothermal amplification
LF	Lateral flow
LPS	Lipopolysaccharide
MDA	Multiple displacement amplification
MIF	Micro immunofluorescence
MOMP	Major outer membrane protein
NAAT	Nucleic acid amplification test
NASBA	Nucleic acid sequence-based amplification
PCR	Polymerase chain reaction
POC	Point-of-care
POCT	Point-of-care testing
RB	Reticulate body
RCA	Rolling circle amplification
RIP	Radioimmuno- precipitation
RPA	Recombinase polymerase amplification
SDA	Strand- displacement amplification
STD	Sexually transmitted diseases
TMA	Transcription mediated amplification

INTRODUCTION

Chlamydia trachomatis is the most prevalent sexually transmitted bacterial pathogen among young people. In men, it causes urethritis, and can induce infection of the prostate gland, testicles and can even result in infertility or sterility. In woman, it is associated with different reproductive tract diseases such as cervicitis and endometritis, and can lead to severe consequences during pregnancy, such as abortion, ectopic pregnancy, or neonatal conjunctivitis/ pneumoniae. The main problem is that most *Chlamydia trachomatis* positive patients are asymptomatic and therefore remain untreated, which, over time, leads to serious complications.

Accurate detection is a prerequisite for efficient diagnosis and effective treatment, and plays a key role in preventing the spread of sexually transmitted pathogens. Various nucleic acid based techniques have recently been developed to detect pathogens from biological samples. Although offering several advantages over traditional detection methods, PCR based diagnostic techniques have some limitations. These include the possibility of accidental contamination by the amplified product due to the high sensitivity, high marginal costs, low inhibitor tolerance, and the need for technically skilled personnel. These limitations have given birth to alternative methods such as isothermal nucleic acid amplification methods.

Isothermal nucleic acid amplification methods offer significant advantages over PCR based methods because they do not require expensive and sophisticated apparatus. Some of these methods can detect nucleic acids directly within biological samples because they are able to tolerate the inhibitors present in biological samples. The list of isothermal nucleic acid amplification tests (NAATs) include transcription-mediated amplification (TMA), nucleic acid sequence-based amplification (NASBA), strand displacement amplification (SDA), rolling circle amplification (RCA), loop-mediated isothermal amplification (LAMP), isothermal multiple displacement amplification (MDA), recombinase polymerase amplification (RPA), and helicase-dependent amplification (HDA), among others.

Here, we describe the application of two isothermal nucleic acid amplification technologies, RPA and LAMP, for the direct detection of the pathogen *Chlamydia trachomatis* from urine samples. Molecular assays based on these amplification methods could provide a good alternative to PCR, especially when utilizing crude biological samples without prior nucleic acid purification steps. These methods can be performed at constant temperature, have high sensitivity and can recognize even a few copies of the template DNA. Furthermore, the detection of an amplification signal can be performed within 10–20 min. The visualization of results can also be performed using lateral flow detection strips because the amplified product could be detected without prior purification. The approaches presented herein could provide a good basis for developing a point-of-care (POC) diagnostic platform in laboratories with limited resources.

1. LITERATURE OVERVIEW

The rate at which sexually transmitted diseases are spreading around the world makes them a major public health concern because of their potential for severe long-term consequences in reproductive health (Apari et al., 2014). Microorganisms such as bacteria, parasites, yeast, and viruses cause sexually transmitted infections (STIs). The most common STIs are caused by *Chlamydia trachomatis*; Herpes simplex virus; *Neisseria gonorrhoeae*; Human immunodeficiency virus; Human papillomavirus; and *Trichomonas vaginalis*. STIs affect both men and women, however, they tend to cause more severe health problems for women. Persistent infections can have serious negative consequences, such as pelvic inflammatory disease, infertility, or permanent damage of a woman's reproductive organs. The main problem with these pathogens is that infection is often asymptomatic, which hinders both diagnosis and treatment (Farley et al., 2003). For that reason, early detection of the pathogens is crucial to initiating appropriate outbreak control measures.

Nucleic acid amplification technologies (NAATs) offer the most sensitive methods of detecting various sexually transmitted pathogens in a laboratory due to their improved sensitivity over immunoassays and traditional culture-based methods (Centers for Disease & Prevention, 2014; Iwamoto et al., 2003; Jevtusevskaja et al., 2016; Kim et al., 2011; Krolov et al., 2014; Lamhoujeb et al., 2009; Van Dyck et al., 2001). In an attempt to reduce the sample processing time and overall cost of PCR- based methods, isothermal nucleic acid amplification methods offer significant advantages because they do not need a thermocycling machine and can be performed with reduced time while providing accurate and reliable results with less-invasive clinical samples for screening (Edwards et al., 2014; Fraczyk et al., 2016; Hu et al., 2010; Krolov et al., 2014).

This dissertation focuses on the detection of *Chlamydia trachomatis* using loop-mediated isothermal amplification and recombinase polymerase amplification methods directly from clinical samples. These restrictions ensure that the resulting methods could be used to analyze clinical samples and possibly be integrated into POC tests.

1.1 Epidemiology of sexually transmitted infections

Sexually transmitted infections may cause epidemics and thereby present a risk to global health and the economy. More than one million sexually transmitted infections are acquired every day worldwide. It is estimated that at least 357 million new infections of chlamydia, gonorrhoeae, syphilis, or trichomoniasis are transmitted each year (Newman et al., 2015). These most common sexually transmitted infections can cause cervicitis, urethritis, and genital ulceration. In addition, they can also lead to more severe complications, including pelvic inflammatory disease, ectopic pregnancy, premature delivery, infertility, neonatal

death, chronic pelvic pain and neurological/ cardiovascular disease in adults, blindness and increased risk of HIV acquisition and transmission (Global Burden of Disease Study, 2015). However, quantifying the prevalence of these infections could provide a good basis for accurate screening, diagnosis, prevention, and management strategies.

In year 2013, 52 995 *Neisseria gonorrhoeae* cases were reported in 28 EU/ EEA countries with an overall rate of 17 per 100 000 population. Gonorrhoeae occurs three times more often in men than in women with a rate of 29 per 100 000 in men and 9,7 per 100 000 in women. The largest proportion of reported cases, 43% were among men who have sex with men; 39% of positive cases were reported among young adults (15–24 years) (European Centre for Disease Prevention and Control, 2015).

Syphilis infection were reported from 29 EU/EEA countries in 2013 with a total of 22 237 cases with overall rate of 5,4 per 100 000 population (European Centre for Disease Prevention and Control, 2015). Syphilis is caused by the bacterium *Trepenoma pallidium* depending in which of the four stages it presents (primary, secondary, latent, and tertiary) (Singh et al., 1999). Like a gonorrhoeae infection, syphilis is more common in men than in women (with a rate of 8,4 per 100 000 in men and 1,6 per 100 000 in women). 58% of these syphilis cases were reported in men who have sex with men and 14% of these cases occur among young people (15–24 years). It may also be transmitted from mother to baby during pregnancy or at birth, resulting in congenital syphilis (Arnold et al., 2000). In 2007, based on World Health Organization (WHO) data, two million syphilis infections are detected among pregnant women annually, 65% of which result in adverse pregnancy outcomes. In 2012, over 900 000 pregnant women were infected with syphilis which resulted in approximately 350 000 adverse birth outcomes, including stillbirth (Newman et al., 2013).

Trichomonas vaginalis is the most common nonviral cause of STI worldwide. In most cases it is asymptomatic in men and cause vaginitis in women (30% of women can also be asymptomatic). It is estimated that between 170–190 million new *T. vaginalis* infections occur worldwide each year. Young age is not a risk factor for trichomoniasis because the infection appears to increase with age for both males and females (Ginocchio et al., 2012; Zhang et al., 1995) at 8.1 % for women and 1.0 % for men (World Health Organization, 2001).

Chlamydia trachomatis genital infection is the most common reported sexually transmitted bacterial infection. More than 100 million cases of *C. trachomatis* STIs arise annually (World Health Organization; 2011), however, this infection can be successfully cured with an effective regimen of antibiotic (such as azithromycin or doxycycline) and prompt treatment. When left untreated, however, as with the aforementioned pathogens, it can be a cofactor for HIV infection (Cohen et al., 1997). In Europe, the number of reported *C. trachomatis* infections has gradually increased since 2004. The increased number of diagnosed cases is attributed to more widespread testing of asymptomatic people using highly sensitive nucleic acid amplification tests (NAATs). In 2013,

384 555 cases of *C. trachomatis* were reported to the European Centre for Disease Prevention and Control (ECDC) from 26 European Union (EU) and European Economic Area (EEA) countries, with a total of 182 documented cases per 100 000 population, hovewer the true incidence and prevalence rates are likely to be significantly higher (European Centre for Disease Prevention and Control, 2013). An ECDC surveillance report estimated that 67% of all reported *C. trachomatis* positive patients in 2013 were in young adults aged between 15–24 years with a higher rate in women aged 20–24 years (1717 cases per 100 000). The estimated prevalence of *C. trachomatis* in four EU/EEA Member states in sexually experienced adults aged 18–26 years ranges from 3–5,3% in women and 2,4–7,3% in men (Redmond et al., 2015). When a general population analysis (without restrictions on age, sexual experience) was performed, the estimated prevalence of *C. trachomatis* in women ranged from 1,1% in Norway to 6,9% in Estonia. In men, it ranged from 0,4% in Germany to 6,2% in Norway (ECDC). All this together clearly demonstrates that the prevalence of *C. trachomatis* varies by age, sexual experience and geographic coverage. Still there is significant variation across the EU and EEA Member states where incidence of diagnosed *C. trachomatis* cases can range from below one to more than 500 cases per 100 000 population. This can be due to the differences in the surveillance systems and detection methods used and the number of people tested.

1.2 Chlamydia trachomatis

Chlamydia trachomatis is a small gram-negative obligate intracellular parasite of eukaryotic cells and is the leading cause of sexually transmitted diseases among young people worldwide. *C. trachomatis* infects not only the columnar epithelium of the cervix, urethra, and rectum, but also non-genital sites such as lungs or eyes. *C. trachomatis* has a rigid cell wall of lipopolysaccharides but typically does not contain bacterial peptidoglycan (Ghuysen et al., 1999). Its circular genome consists of 1 042 519 nucleotide base pairs (58.7% A+T) and has approximately 894 putative protein encoding genes. *C. trachomatis* strains contain a 7493 base pair extrachromosomal multiple cryptic plasmid, which is conserved among different serovars and strains (Pickett et al., 2005).

Three different *Chlamydia trachomatis* biovars based on the major outer membrane protein (OmpA) are associated with different clinical manifestations (Brunelle et al., 2006).

The first group contains serovars Ab, B, Ba and C that typically infect epithelial cells from the conjunctival mucosa and cause trachoma – a chronic eye disease. Globally, it affects poor countries that have little or no healthcare and causes infectious blindness.

The second group consists of serovars D–K, which all cause genital tract infections such as pelvic inflammatory disease, ectopic pregnancy, and urethritis, and can lead to neonatal pneumonia/conjunctivitis. Although *C. trachomatis*

genital strains are frequently asymptomatic for long periods of time, undetected and untreated infections result in persistent transmission and is one of the major causes of serious complications such as epididymitis in men; pelvic inflammatory disease, ectopic pregnancy, or even infertility in women.

The third group, which includes serovars L1-L3, cause genital ulcer disease such as lymphogranuloma venereum. It relies on their ability to infect mononuclear phagocytes and then spreads into regional lymph nodes. If left untreated, however, these serovars are able to cause more severe consequences such as proctitis, rectal strictures, and lymphatic obstruction.

1.2.1 *Chlamydia trachomatis* developmental cycle

The *C. trachomatis* biphasic developmental cycle involves tight interaction between the pathogen and host cells during which the bacterium exist in two life cycle forms – infectious metabolically inactive elementary body (EB) and replicating reticulate body (RB), and are distinguished by morphological and biological properties (Abdelrahman et al., 2005). The extracellular form of an elementary body (EBs) is highly infectious, dispersal and analogue to spores. They are small, approximately 0,3 μm diameter, have a highly compacted nucleoid and rigid cell wall, which allows it to survival in the extracellular environment. Once it enters epithelial cells via an inclusion vacuole (by endocytosis), it transforms into the reticulate body (RBs) – non-infectious, metabolically active form, which is able to replicate and accumulate within cytoplasmic inclusions within the cell. This differentiation process can be blocked by adding antibiotic inhibitors (Scidmore et al., 1996). RBs are usually larger than EBs (1 μm diameter), and contain relax chromatin and are sensitive to osmotic lysis. The reticulate bodies then transform into resistant elementary bodies and are released from the lysed, ruptured cell. The free bacteria then target neighboring cells to continue the replication process **(Figure 1).**

There is less information about the mechanisms and signals that regulate the intracellular development of *C. trachomatis*. However, it has been shown that *C. trachomatis* goes through three different gene expression stages associated with changes in its developmental cycle (Albrecht et al., 2010; Belland et al., 2003; Nicholson et al., 2003).

During development stage one, gene expression starts within 6–12 hours after infection. During this stage, the genes responsible for basic cellular functions, transport of nutrients, and starting intracellular infectious as well as midcycle genes responsible for cell division and growth are expressed.

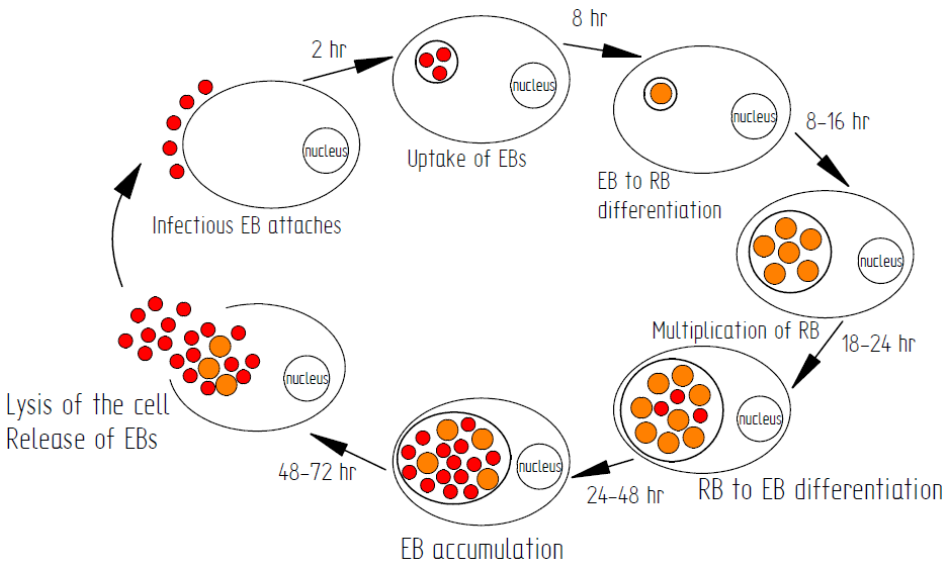


Figure1. *Chlamydia trachomatis* biphasic developmental cycle. Infection is initiated by an extracellular environmentally resistant, metabolically inactive and nonreplicating infectious form termed elementary bodies (EB) that attach and enters mucosal epithelial cells (after 2 hour). After internalization, EB form in inclusion, where EB transforms into a larger replicative form – reticulate bodies (RB) (8–18 hours). After 18 to 24 hours, RB transforms back into the EB form and after 48–72 hours release themselves from inclusion vacuoles. The newly released infectious EB can now infect neighboring cells.

Development stage two starts 18 hours after the beginning of the infection. This time corresponds to the point when the production of RB is maximal. Development stage two genes include those responsible for energy metabolism, cell envelope biogenesis, secretion systems, protein folding and different DNA modification processes. Some of these genes are also thought to be involved in both the differentiation of RB to EB and DNA condensation.

Developmental stage three includes late and very late transcripts responsible for EB formation and condensed nucleoid formation.

1.2.2 Targets of *C. trachomatis* for molecular assays

Various targets have been used to detect *Chlamydia trachomatis*, however, the most attractive is the cryptic plasmid and the major outer membrane gene (MOMP) because these targets provided the best sensitivity and specificity and are widely used for the molecular diagnosis of *Chlamydia trachomatis*, including on commercial basis (Garland et al., 2000).

The cryptic plasmid of *Chlamydia trachomatis* is a multiple extrachromosomal target present in approximately 7–10 copies per cell and is highly conserved among different *C. trachomatis* strains. The cryptic plasmid is used as a

target in the following commercially available amplification techniques that amplify specific *C. trachomatis* sequences in the cryptic plasmid: Abbott RealTime CT/NG assay; BD ProbeTec ET(QX) CT/GC Amplified DNA assay; Cobas CT/NG test; and the Illumigene CT/NG assay.

The second widely used genomic target is a single copy, highly variable ompA (MOMP) gene which is used both in culture and non-culture methods for *C. trachomatis* detection as well as in the commercially available COBAS® TaqMan® CT (dual-target detection strategy – cryptic plasmid DNA and MOMP gene genomic DNA) assay. The nucleic acid hybridization assay Hybrid Capture 2 (HC2) also detects the ompA gene, as well as the cryptic plasmid of *C. trachomatis*.

There are several commercially available assays that amplify multicopy ribosomal RNA (rRNA; 16S and 23S) such as Aptima Combo 2 assay and Aptima CT assay as well as hybridization assay Gen-Probe PACE2.

1.2.3 Detection methods of *Chlamydia trachomatis*

1.2.3.1 Culture method

The cell culture method was considered the gold standard for *Chlamydia trachomatis* detection until the development of NAATs. Currently, due to the minimal potential for contamination, high specificity (near 100%), and ability for isolation, cell culture continues to be the gold standard for medicolegal issues such as sexual assault and child abuse (Centers for Disease & Prevention, 2014). Besides urethral specimens (women and asymptomatic men), the recommended specimen types for culture method are: nasopharyngeal (infants), conjunctival, rectal, and vaginal specimens (prepubertal girls). The culture method has special requirements for sample collection and storage. Collected samples should be stored in a specially design medium to maintain the viability of *C. trachomatis* at appropriate temperatures (≤ 4 °C) within 24 hours. If the time between collection and processing of the sample is more than 24 hours, the specimen should be frozen at –70 °C. However, this is less advisable because it diminishes the viability by at least 20% (Mahony et al., 1994; Reeve et al., 1975). Specimens are then inoculated onto a confluent cell monolayer (McCoy; HeLa 229; Buffalo Green Monkey Kidney cells) to maintain the growth of *C. trachomatis*. After 48–72 hours of growth, intracytoplasmatic inclusions develop that contain *C. trachomatis* EBs and RBs. Viable chlamydial elementary bodies can then be fluorescently labeled by antibodies that bind to chlamydial lipopolysaccharides (LPS) or major outer membrane protein (MOMP) for specific *C. trachomatis* detection (Lees et al., 1988). Iodine or Giemsa staining are not recommended due to their poor sensitivity and specificity (Hammerschlag et al., 1999).

Moreover, culture based methods allow one to use the organism to perform additional experiments such as sensitivity to antibiotics or serovar identification.

However, other tests for *Chlamydia trachomatis* detection are required due to several disadvantages of the culture method:

1) time – consuming and labor intensive – the result can be obtained only after 3–7 days (EB visualization after 48–72 hours) in specialized experienced laboratories
2) to obtain a viable organism, special transport media are needed
3) requirement of stringent storage temperature
4) expensive
5) variation among laboratories in performance and
6) relatively insensitive (50–85% compared with NAATs) (Black, 1997)

1.2.3.2 Nonculture, non-nucleic acid amplification methods

The first nonculture and non-nucleic acid amplification tests for *C. trachomatis* detection included antigen based detection tests – Enzyme Immunoassay test (EIA) and Direct fluorescent antibody tests (DFA).

The EIA method is based on the immunochemical detection of *C. trachomatis* specific lipopolysaccharides (LPS) using monoclonal or polyclonal antibodies that are labeled with an enzyme (Black., 1997). The colored product is then measured with a spectrophotometer (or can be also detected by a fluorescence reader). The manufacturer's instructions should be followed when EIA specimen are collected, stored and processed. This method could be applied on cervical, urethral, rectal, pharyngeal, conjunctival and urine samples. Most EIA are less sensitive than the culture method, however commercially available EIAs provide improved sensitivity/ specificity relative to culture because they include a secondary blocking reagent for result verification (86% sensitivity and 95% specificity) (Moncada et al., 1994). Several rapid point-of-care tests based on EIA technology has been developed, however, these display less sensitivity (52%) and specificity (95%) as compared with EIA performed in the laboratory (Lauderdale et al., 1999). The main disadvantages of EIA are that it can give false positive results (cross-reaction with other bacterial LPS), cannot differentiate among different chlamydia species, are time- consuming (3–4 hours for sample processing), and all positive EIA results must be confirmed.

DFA visualizes chlamydia elementary bodies by staining them with specific antibodies for MOMP/LPS and identifies these using fluorescent microscopy. This method has been used to detect infections from conjunctival, urethral, rectal, pharyngeal specimens, however it is preferably used for endocervical smears. The overall sensitivity of DFA is 80–90% and specificity 98–99% (Chernesky, 2005; Chernesky et al., 1986; Quinn et al., 1987) relative to the culture method and approximately 70% compared with NAATs. DFA is also the only diagnostic test that allows for the simultaneous evaluation of specimen adequacy by visualization of cuboidal columnal epithelial cells. At the same time, this method is laborious and requires highly skilled personnel, and is thus

not suitable for a large number of specimens. DFA is mainly used for confirmation of positive results of nonculture tests such as EIA (Chan et al., 1994; Kellogg et al., 1993; Black, 1997).

Two nucleic acid hybridization assays have been approved by the FDA to detect specific sequences of DNA complementary to *C. trachomatis* sequences that are present in the sample: Gen-Probe PACE 2 and Digene Hybrid Capture II assay. The nucleic acid hybridization test PACE 2, which allowed hybrid (DNA:rRNA) detection using a luminometer, was commercially available until the year 2012. This tests showed more sensitive *C. trachomatis* detection as compared with antigen detection because it targeted a multiple copy of 16 S rRNA (Lees et al., 1991). The Digene Hybrid Capture II assay utilizes RNA probes that are specific to the *C. trachomatis* cryptic plasmid sequence as well as genomic DNA. This test displayed higher sensitivity compared to the PACE2 test (Modarress et al., 1999).

There are also several antibody detection tests based on serological techniques: complement fixation tests (CFT), radioimmuno-precipitation (RIP) and micro immunofluorescence (MIF). These tests were mainly used to distinguish between acute and chronic infectious, however, the accuracy and reliability of these techniques are questionable. These tests are time-consuming, labor-intensive and are not recommended for use because they might produce false-negative results due to the inability of a *C. trachomatis* reinfection to bring out an antibody response.

1.2.3.3 Nucleic Acids Amplification Tests (NAATs)

Recently developed nucleic acid amplification techniques provide rapid, sensitive and efficient diagnosis of *C. trachomatis*. In comparison with traditional culture methods or antigen/antibody based techniques, these tests provide high sensitivity (detecting as little as a single target gene copy) and specificity, and significantly simplify the pathogen detection process (Centers for Disease & Prevention, 2014). Compared with the culture method, these tests do not require viable pathogens and can detect *C. trachomatis* also from vulval, vaginal, urine, or semen specimens. Current NAATs are based on polymerase chain reaction (PCR) and are widely used for clinical *C. trachomatis* diagnostics. Various commercially available amplification tests are available, such as the Abbot RealTime assay or the Roche Cobas Amplicor assay, which amplifies a *C. trachomatis* sequence in the cryptic plasmid that is found in more than 99% of *C. trachomatis* strains. The Cobas Amplicor assay also amplifies the ompA (MOMP) gene of *C. trachomatis*. Results can be obtained within an hour, which allows for prompt diagnosis and rapid treatment. The sensitivity of these assays is 85–98% and specificity is 95–99.9%, depending on the assay and gold standard utilized (Schachter, 1999). Internal controls that can be incorporated into NAATs enables one to identify inhibitory substances in the sample that may produce false-negative results (Betsou et al., 2003).

Although NAATs are highly sensitive and specific, they still require trained personnel, expensive apparatus, and complicated sample preparation techniques. This effectively restricts their use to centralized hospitals.

1.3 Isothermal nucleic acid amplification methods

Isothermal DNA amplification methods can provide a good alternative to PCR-based techniques (Francois et al., 2011). The major isothermal amplification methods in use include: nucleic acid sequence-based amplification (NASBA) (Deiman et al., 2002), loop-mediated isothermal amplification (LAMP) (Notomi et al., 2000), strand-displacement amplification (SDA) (Walker et al., 1992), rolling circle amplification (RCA) (Ali et al., 2014), helicase-dependent amplification (HDA) (Vincent et al., 2004), and recombinase polymerase amplification (RPA) (Abd El Wahed et al., 2015). All isothermal amplification methods differ from each other in terms of reaction duration, performance temperature, template utilization, numbers of primers/ enzymes, tolerance to inhibitors, and detection of the product and mechanism.

NASBA, also known as 3SR or transcription mediated amplification, is an isothermal transcription-based amplification method specifically designed to amplify a single-stranded RNA sequence at 41 °C within 90–120 min (Gill et al., 2008; Guatelli et al., 1990). NASBA uses two RNA target-specific primers and three enzymes (avian myeloblastosis virus reverse transcriptase, T7 DNA-dependent RNA polymerase (DdRp) and RNase H). The product can be detected using enzyme-linked gel assay, electrochemiluminescence, by lateral flow, or with molecular beacon technology (Connelly et al., 2008; Nugen et al., 2009; van Gemen et al., 1994).

SDA utilizes four different primers and two enzymes (DNA polymerase 1 (exo-Klenow) and HincII exonuclease) for dsDNA template recognition and is the basis for commercial BDProbeTec detection tests. Typically, amplification takes places at 37 °C within 120 min. The main limitation of the SDA method is that it is not able to efficiently amplify long target sequences (Walker et al., 1993). SDA product detection can be performed using the gel electrophoresis or real time monitoring (Nadeau et al., 1999).

RCA amplifies a circular DNA template using a strand displacing DNA polymerase and one primer at 37 °C within 60 min. Target detection can be accomplished using gel electrophoresis, real-time monitoring or colorimetric method (Chen et al., 2014; Hamidi & Ghourchian, 2015).

HDA utilizes DNA helicase to produce single-stranded templates for two primer hybridization and subsequent primer extension by a DNA polymerase (Vincent et al., 2004). The entire procedure is performed at 65 °C within 75 – 90 min. The HDA amplicons can be detected using gel electrophoresis, real-time format, and ELISA (Kivlehan et al., 2011).

All of these isothermal amplification methods can greatly accelerate target amplification and are performed at a single constant temperature, without the

need for a thermocycling machine, which significantly simplifies the process. Also, some of these isothermal nucleic acid amplification methods show good tolerance to the inhibitors usually found in clinical samples and thus eliminate the need for prior DNA purification (Fraczyk et al., 2016; Iwamoto et al., 2003; Jevtusevskaja et al., 2016; Krolov et al., 2014; Mukhopadhyay et al., 2012; Poon et al., 2006; Wozniakowski et al., 2014). These methods also do not require sophisticated equipment, which makes them a good diagnostic tool for point-of-care diagnostics, especially in resource poor countries.

In this section, I will more precisely describe both the LAMP and RPA isothermal amplification methods for *C. trachomatis* detection and will highlight their application in molecular diagnostics. **Table1** shows the characters of the main isothermal amplification methods that are used to detect *Chlamydia trachomatis*.

Table 1. Isothermal amplification methods for detection of *C. trachomatis*

Method	**Detection limit/Sensitivity**	**Detection strategy**	**Sample type**	**Sample pretreat-ment**	**Target**	**Viide**
LAMP	100 ng DNA within 10–30 min	Naked eye, gel electrophoresis	Cervical swab	Extracted DNA	ompA	(Choopara I & Sombonna N, 2014)
	0.04 pg (4500 copies) of DNA within 45 min (95% specificity, 90– 100% sensitivity)	Colorimetric detection (Hydroxynaphthol blue	Endo-cervical swab	Heat at 95°C 5 min	ompA	(Choopara et al.,, 2017)
LAMP	capable of detecting 5 theoretical copies of target material under ideal conditions, was not quantitative below 1000 copies (within 45 min)	Real –time monitoring	Cervical/ vaginal/ urine samples + ATCC 33530D DNA	Extracted DNA	cryptic plasmid	(Nixon et al., 2014)
LAMP	10 copies per reaction	Colorimetric detection, gel electrophoresis	Vaginal swab	Solid-phase DNA extraction	NA	(D. J. Shina 2013)
LAMP	55 copy within 1 hour (95,6% sensitivity, 100% specificity)	Real-time bioluminiscent	Urine; swab	Extracted DNA	cryptic plasmid	(Gandelman et al., 2010)
LAMP	ten copies of the CT target per reaction within 22–30 min	Real time monitoring	–	heat	cryptic plasmid	(G. Xu, et al., 2015)

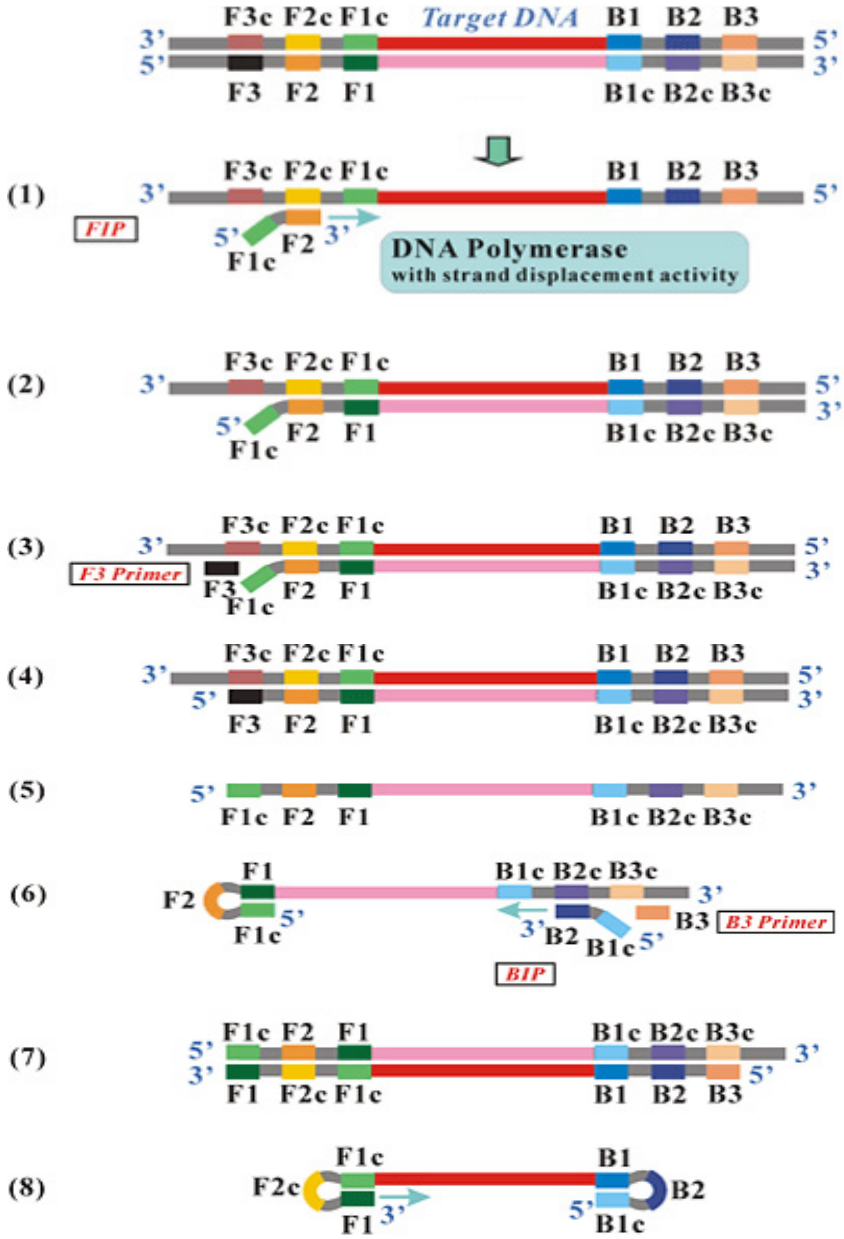
Method	Detection limit/Sensitivity	Detection strategy	Sample type	Sample pretreat-ment	Target	Viide
LAMP	25 pasmid copies (73% sensitivity, 100% specificity) within 21 min	Real time monitoring; lateral flow dipsticks	urine	Anti-microbial peptide lysis	cryptic plasmid	(Jevtusevs-kaja et al., 2016)
RPA	50 plasmid copies (83% sensitivity, 100% specificity) within 20 min	Lateral flow dipsticks	urine	Heat 90° C 5 min	cryptic plasmid	(Krolov et al., 2014)
Gen-Probe TMA assay	(sensitivity 76–93,8%, specificity 99,3–100%)[1] (sensitivity 88,6–95,6%, specificity 98,7–99%)[2] (sensitivity 88–100%, specificity 98,7–99,5%)[3] (sensitivity 93,2%, specificity 99%)[4] sensitivity 92%, specificity 99,6%)[5] Amplification 1 hour	luminometer	Women Urine[1] Male urine[2] Endocervi cal swabs[3] Male urethral swabs[4] Vulvar[5]	Manu-facture protocol (Heating+ hybridi-zation)	rRNA	(Pasternack et al., 1997) (Stary et al., 1998) (Crotchfelt et al., 1998) (Pasternack et al., 1999)
SIBA	20 EBs within 35–60 min	Real-time monitoring	NA	Heat 90° C 10 min	cryptic plasmid	(Eboigbodin et al., 2016)
HDA	25 EB/ml; 0,05IFU/assay Within 120 min (sensitivity 92,9–100%; specificity 98,3–100%)[6] (sensitivity 92,9%; specificity100%)[7] (sensitivity 94,7%; specificity100%)[8]	Real-time monitoring	Urine[6] Vaginal swab[7] Cervical swab[8]	Lysis + DNA denatu-ration + hybridi-zation	ompA, cryptic plasmid	(O'Neil et al., 2011) (Doseeva et al., 2011)

Method	Detection limit/Sensitivity	Detection strategy	Sample type	Sample pretreat-ment	Target	Viide
NASBA	0,001 IFU[6] 1IFU[5,7]	Nothern Blot; enzyme-link gel assay (ELGA)	Cervical scrapings, urine	Lysis + silica base method for DNA/RNA purification	cryptic plas-mid[5]; 16 SrRNA[6] ompA[7]	(Morre et al., 1996; Morre et al., 1998)
NASBA	1 IFU of *C. trachomatis* and 100 RNA molecules of 16SrRNA within 95 min (sensitivity 100% specificity 98,6%; reference culture method)	electrochemilu minescent (ECL)	urethral or cervical swab specimens	Extracted genitou-rinary tract specimens	16 SrRNA	(Mahony et al., 2001)
BDPT-SDA	(sensitivity 76–100%; specificity 99,2–100%)[9]	Real-time fluorescent detection	Cervical, vaginal, urethral swabs, urine, seminal fluid	Manu-facture protocol (lysis)	cryptic plasmid	(Haugland et al., 2010) (Little et al., 1999) (Cosentino et al., 2003) (Fontana et al., 2005)
RAM	10 *C. tracho-matis* EB in less than 2 h (sensitivity 93,3% specificity 100%)	Agarose gel	Endo-cervical specimen	Lysis + hybridi-zation	cryptic plasmid	(Zhang et al., 2002)

1.3.1 Loop mediated isothermal amplification (LAMP)

LAMP is a widely adopted and robust nucleic acid amplification technique first introduced 17 years ago by Notomi and his colleagues. Nowadays, it has become an ideal tool for diagnostics that enables one to detect different viral, bacterial, fungal, and parasitic pathogens (Edwards et al., 2014; Francois et al., 2011; Iwamoto et al., 2003; Kaneko et al., 2005; Mukhopadhyay et al., 2012; Nakao et al., 2010; Notomi et al., 2000; Parida et al., 2008).

In LAMP, the target sequence is amplified at a constant temperature of 60–65 °C using between 4–6 specially designed primers and a polymerase with a strand displacement activity to identify 6 distinct regions on the target gene sequence (**Figure 2)**. Adding a pair of loop primers significantly accelerates amplification by increasing sensitivity and reducing reaction amplification time (Nagamine et al., 2002). This allows one to amplify target DNA with high selectivity and generate an amplification product that contains single-stranded loop regions where primers can bind without template denaturation.


F3c F2c F1c
Target DNA
B1 B2 B3
3'
5'
5'
3'
F3 F2 F1
B1c B2c B3c
(1)
FIP
F1c
F2
DNA Polymerase
with strand displacement activity
(2)
(3)
F3 Primer
F3
(4)
(5)
(6)
F2
B2
B3
B3 Primer
BIP
(7)
(8)

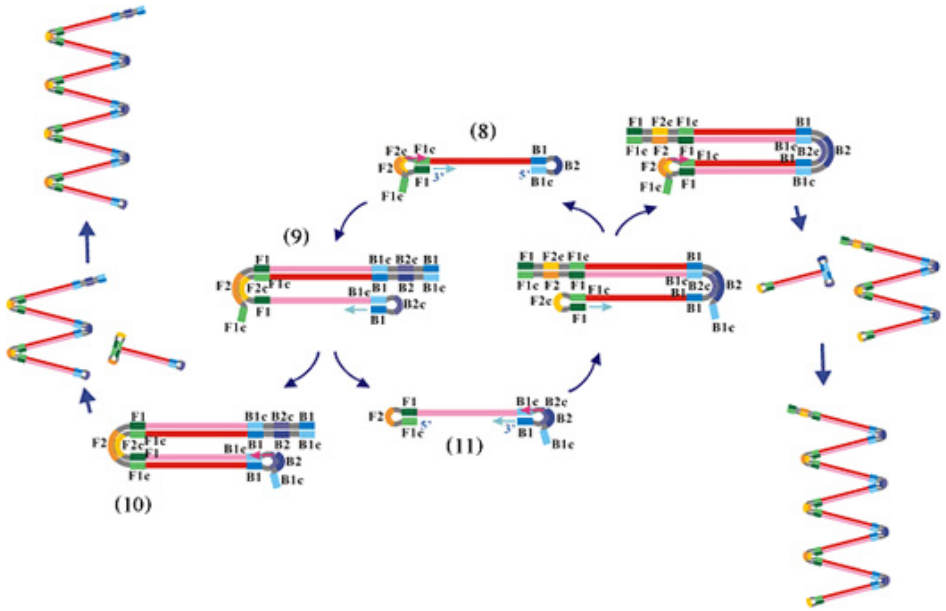


Figure 2. Mechanism of loop-mediated isothermal amplification (Eiken chemical Co. Ltd.). **(1–2)** F2 region of FIP hybridizes to F2c region of the target dsDNA and initiates complementary strand synthesis using DNA polymerase with a strand displacement activity. **(3–6)** The F3 primer hybridizes to the F3c region of the target DNA, extends, displacing the FIP linked complementary strand with a loop at the 5' end. **(6)** Now ss DNA with a loop at the 5' end serves as a template for BIP where B2 region hybridizes to B2c region of the template DNA. Now, B3 hybridizes to B3c region of the target DNA, extends, displacing the BIP linked complementary strand. **(8)** As a result a dumbbell shaped DNA is formed. **(9–11)** The dumbbell shaped DNA is converted to a stem loop structure and serves as an initiator for LAMP cycling when FIP hybridizes to the loop of the stem-loop DNA structure. The releasing strand forms a loop at the 3' end. Then B1 extension release the FIP strand which start to form dumbbell shaped DNA. BIP hybridizes now to these products **(10–11)**. As a result, stem loop DNA with various stem lengths and various cauliflower like structures with multiple loops are formed.

The LAMP method provides high amplification efficiency, with DNA being amplified 10^9–10^{10} times within 15–60 min. Moreover, these amplification methods are less prone to inhibitory substances that usually affect the sensitivity of PCR which allows one to use unprocessed, crude sample thereby simplifying the entire detection process (Edwards et al., 2014; Fraczyk et al., 2016; Iwamoto et al., 2003; Jevtusevskaja et al., 2016). Due to its simplicity and low cost of operation, it is an ideal tool for clinical point-of-care applications where accurate and rapid diagnosis is essential.

1.3.1.1 LAMP product detection/ analysis strategy

The most common way to detect LAMP products is gel electrophoresis. During isothermal amplification, a mixture of various length stem-loop products are formed which are then visualized under UV illumination as a ladder-like pattern. The specificity of the amplification product can further be confirmed by restricting endonuclease digestion. However, this detection method is laborious, time-consuming, needs additional equipment and can lead to carryover contamination.

Alternatively, LAMP amplification products can be directly visualized using DNA binding dyes (e.g. SYBR Green 1; EvaGreen; SYTO 9). In Paper II, we demonstrate that addition of Evagreen into the LAMP reaction mixture prior to amplification allows one to detect the product both by direct visualization under UV illumination or using real-time monitoring. The use of DNA intercalating dyes can also significantly reduce carryover contamination problems caused by aerosol during product analysis process.

During LAMP amplification, a large number of LAMP amplicons are generated, thereby increasing the amount of white precipitate of magnesium pyrophosphate ion released as a product. This can be identified by the presence of turbidity in the reaction mixture both by the naked eye or by real time turbidimeter (Sappat et al., 2011).

Electrochemical measurement of LAMP products is also possible. The integration of electrochemically active DNA binding compounds to the reaction mixture prior to amplification allows one to detect amplified DNA with a low input template DNA (Hsieh et al., 2012).

In Paper II, we present another advance in LAMP amplicon detection technology which uses direct lateral flow dipstick analysis to enable immunochromotographic detection of the labeled product. This detection method allows for the accurate detection of LAMP amplicons and eliminates the need for sophisticated equipment and time-consuming data analysis. Thus, it is an excellent choice for point of care applications.

1.3.2 Recombinase polymerase amplification (RPA)

RPA is another nucleic acid isothermal amplification method that, due to its superiority in speed, sensitivity, specificity and portability, has been widely adapted to detect various bacteria, viruses, parasites, and even genetically modified organisms (Euler et al., 2013; C. Xu et al., 2014). This method was firstly introduced in 2006 by Piepenburg et al (Piepenburg et al., 2006). RPA utilizes three key proteins that operate optimally at 37–42 °C: phage-derived recombinase UvsX, ssDNA binding protein (SSB), and DNA polymerase with strand displacement activity (Sau polymerase from *Staphylococcus aureus*). The RPA reaction is also supported by the presence of accessory proteins: UvsY co-factor and a particular crowding agent (Carbowax20M). **Figure 3** presents a schematic of the RPA working process. Typically, an RPA reaction runs to completion in

5–20 min, depending on the starting template copies per reaction as well as amplicon size. As with LAMP, all steps from amplification to detection can be conducted under isothermal conditions within one reaction tube. Due to its high sensitivity (10^4 fold amplification within 10 min) and specificity, a minimal number of sample processing steps, and available lyophilized format, it can also be widely used in low and middle income countries as a reliable point of care test.

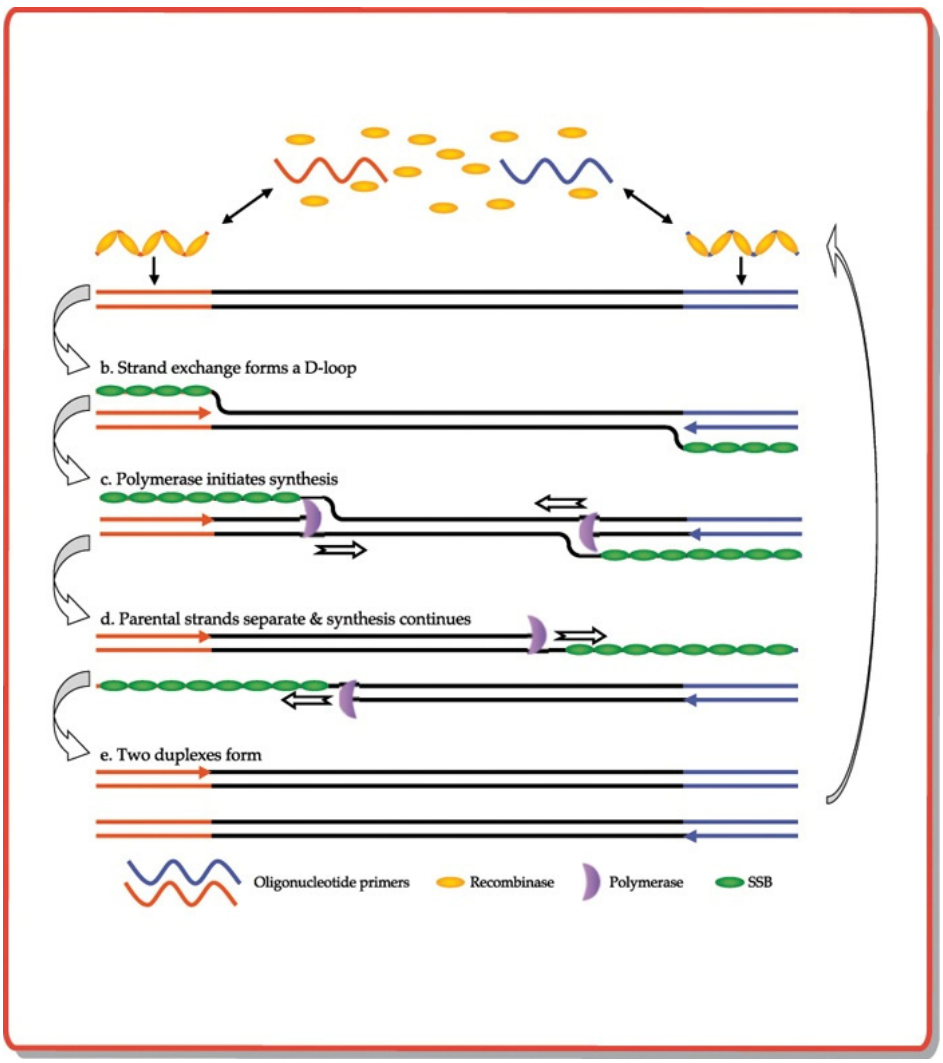


Figure 3. Mechanism of recombinase polymerase amplification (TwistDX). The reaction begins with a recombinase that binds to the primers and together anneal to specific target region. Single stranded binding proteins (SSBs) are then stabilizing separated strands. Polymerase with strand displacement activity continues the amplification.

There are different RPA commercial kits available from TwistDX (Cambridge, UK) for either DNA or RNA amplification that offer different detection strategies, including gel electrophoresis, lateral flow, or real-time fluorescent detection.

1.4 Considerations in biological sample selection and screening

Accurate and prompt diagnosis of *Chlamydia trachomatis* includes collecting the right specimen, transportation, storage, and processing (Centers for Disease & Prevention, 2014). Specimen quality plays a crucial role in determining an adequate result because a lack of urethral or endocervical columnar cells may affect both the sensitivity and specificity of diagnostic tests.

It should be noted that the type of swab used for specimen collection is also an important consideration because it can be toxic to the cell culture or even induce *C. trachomatis* growth inhibition (Mahony et al., 1985). To improve the rate of isolation, some laboratories use cytologic bruches instead of swabs; however they are not suitable to use on pregnant women and can cause bleeding which may inhibit nonculture diagnostic tests (Akane et al., 1994; Moncada et al., 1989). For urine specimes, first-void urine sould be used and sample collection should be delayed until ≥1hour after the patient has voided.

Endocervical, vaginal, vulval, urethral, and rectal swabs and first void urine are the common samples taken from female patients. Urethral and rectal swabs and first void urine sample as well as prostatic or semen fluid can also be collected from male patients. For genital *C. trachomatis* specimens, nucleic acid amplification tests (NAAT) are the recommended and preferred test methods. However, invasive specimens such as endocervical or urethral swabs can also be screened by nonculture/ non-NAAT methods (EIA, DFA, unamplified nucleic acid hybridization test) or may be collected for culturing if isolation is required (e.g., sexual abuse, treatment failure). To detect non-genital *C. trachomatis* sample types such as rectal, pharyngeal, or conjunctival, either the culture method or DFA are preferred (when an isolate is needed) because both show high specificity.

One should also consider other factors when selecting and recommending a screening test, such as the cost of the analysis, additional tests for positive result verification, likelihood of returning a positive screening patient for treatment, and the requirement of a suitable laboratory environment.

1.5 Amplification inhibitors in biological samples and strategies for their removal

PCR inhibitors generally interfere with DNA polymerases or interact with template DNA. Direct binding of agents to single-stranded or double-stranded DNA can prevent amplification and facilitate co-purification of both the inhibitor and DNA. Well-known PCR inhibitors present in human patient samples are plasma, immunoglobulin G, hemoglobin, and lactoferrin – in blood samples; urea and high salt concentrations (K^+, Na^+, Mg^{2+}) – in urine samples; humic acid present in soil; high Ca^{2+} ion, fatty acids and haem concentrations in food samples (Schrader et al., 2012) (Abu Al-Soud & Radstrom, 2000) (Al-Soud et al., 2001). Protease and nuclease activity in blood, urine, and food samples could also contribute to the reduced efficiency of amplification. PCR inhibitors can also interact directly with a thermostable DNA polymerase to block enzyme activity. For example, PCR using Taq DNA polymerase can be completely inhibited in the presence of less than 0.2% blood in the reaction (Abu Al-Soud et al., 2000). DNA polymerases have cofactor requirements that can also be a target of inhibition. Magnesium is a critical cofactor, and agents that reduce Mg^{2+} availability or interfere with binding of Mg^{2+} to the DNA polymerase can inhibit PCR.

The most common method used to remove amplification inhibitors from samples is DNA purification. Although it is very efficient, DNA purification can be an expensive and complicated procedure, and is hard to apply in POC settings. Various techniques and agents are utilized to overcome inhibitory effects from crude patient samples. For example, it was found that the addition of betaine, bovine serum albumin, single-stranded DNA-binding protein of the T4 bacteriophage (gp32), or a cocktail of protease inhibitors can partially reduce the inhibition from blood samples and can allow Taq DNA polymerase to work in up to 2% blood present in the reaction (Topal et al., 1983) (Kreader et al., 1996). Dilution of the sample, heat treatment, treatment with activated carbon, addition of Tween 20, DMSO, PEG 400, ß-mercaptoethanol, dithiothreitol have also been applied to reduce the effects of inhibitors (Schrader et al., 2012).

However, the selection of resistant DNA polymerase is the most effective of all techniques targeted to reduce amplification inhibition. For example, an N-terminal deletion of Taq DNA polymerase is 10–100-fold inhibition resistant to whole blood compared to full-length, wild-type Taq (Kermekchiev et al., 2009). Furthermore, an additional mutation at codon 708 further enhances the resistance of Taq to various inhibitors of PCR reactions, including whole blood, plasma, hemoglobin, lactoferrin, serum IgG, soil extracts and humic acid, as well as high concentrations of intercalating dyes (Kermekchiev et al., 2009). It was shown that blood PCR inhibitors predominantly reduce the speed of DNA extension by the polymerase (Kermekchiev et al., 2009).

While PCR has been extensively studied in terms of sample inhibition, little is known about crude sample effects on isothermal amplification methods. There are some indications that isothermal nucleic acid amplification methods

are less sensitive towards biological sample components (Francois et al., 2011; Kaneko et al., 2007; Krolov et al., 2014). For example, unprocessed urine normally requires DNA extraction prior to PCR amplification, because urea inhibits PCR at concentrations above 50 mM, and the average concentration of urea in human urine is 330 mM (Khan et al., 1991). Loop-mediated isothermal amplification (LAMP), on the other hand, tolerates significantly higher urea concentrations of ≤1.8 M (Edwards et al., 2014). Several reports claim potential applicability of isothermal amplifications, such as LAMP, recombinase polymerase amplification (RPA), for molecular diagnostics directly from crude samples, such as urine and blood (Fraczyk et al., 2016; Kohda et al., 2014; Krolov et al., 2014) (Hill et al., 2008). Thus, application of isothermal NAAT allows one to skip the nucleic acid purification steps, which are crucial for POC applications.

2. AIMS OF THE STUDY

The main objective of the work presented in this dissertation was to evaluate two isothermal amplification methods – LAMP and RPA in an attempt to find a more sensitive, robust, and cost effective isothermal amplification based molecular assay suitable for point-of-care screening of pathogens directly from unprocessed urine. These finding can help to avoid the need for long and tedious genomic DNA purification steps prior to amplification. We also tried to improve these isothermal amplification methods by using different pathogen lysis strategies to find the best way of releasing genomic DNA and by utilizing different DNA polymerases to find urine tolerant polymerases. The main objectives of this study are described below.

- **Paper I:** The main aim was to develop an RPA based assay for rapid, highly sensitive and specific detection of *C. trachomatis* directly from urine samples suitable for application as a point-of-care test.

- **Paper II:** The main aim was to apply the LAMP-based assay with an antimicrobial peptide lysis mix directly on clinical samples.

- **Paper III:** This paper explores additional possibilities for further improvement of isothermal amplification methods by utilizing already existing strand displacement polymerases. Because DNA polymerases are the key enzymes that possess the amplification reaction we investigated how urine sample components can affect amplification efficiency to select more rapid and urine tolerant polymerases.

3. MATERIALS & METHODS

The methods used in this dissertation are described in detail in each publication. This chapter provides only a brief description of each method used.

3.1 LAMP reaction

Loop-mediated Isothermal Amplification (LAMP) reaction was performed according to the protocol supplied by Eiken Chemical Co Ltd with a reaction volume of 50 µl. In our experiments, we adapted loop-mediated isothermal amplification for real-time detection by adding EvaGreen and ROX fluorescent dyes and removing biotin/FAM labelling from the primers (the concentration of all other components remained unchanged from the traditional LAMP reaction) **(Table 2)**.

Table 2. Protocol for qLAMP

	Stock concentration	Final concentration	Dilution factor	Amount per one reaction (50 µl)
F3 primer	100 µM	0,2 µM	50x	0,1 µl
B3 primer	100 µM	0,2 µM	50x	0,1 µl
FIP primer	100 µM	1,6 µM	6,25x	0,8 µl
BIP primer	100 µM	1,6 µM	6,25x	0,8 µl
LF primer	100 µM	0,8 µM	12,5x	0,4 µl
LB primer	100 µM	0,8 µM	12,5x	0,4 µl
MgSO4	25 mM	6 mM	4,167x	12 µl
dNTP	25 mM	1.4 mM	17,86x	2,8 µl
10x polymerase buffer	10x	1x	10x	5 µl
Betaine	5M	0.8 M	6,25x	8 µl
Bsm polymerase	8 U/µl			2 µl
EvaGreen	20x	0.2x	100x	0,5 µl
ROX				0,3 µl
MQ water				11,8 µl
(Standard DNA or sample)				(5 µl)
			TOTAL	50 µl

3.2 RPA reaction

The protocol for the RPA reaction are provided in **Table 3** and the general layout of the *C. trachomatis* detection assay in **Figure 4**. The forward RPA primer was labeled with biotin and the reverse RPA primer with 6-carbo-xyfluorescein (FAM), thereby enabling biotin-FAM double-labeled product detection on the lateral flow strips. RPA reaction products were diluted 500 times in a dilution buffer and analyzed on PCRD-2 strips (Forsite Diagnostics, York, UK) or purified and resolved on agarose gel. For the RPA product specificity analysis, restriction digests were performed using either PmlII restrictase (for *C. trachomatis* amplification product) or HphI restrictase (for H. sapiens GAPDH amplification product) according to the manufacturer's protocol.

Table 3. Conditions and steps of the RPA reaction

Step no.	Description
1	Prepare the rehydration solution. Vortex and spin briefly: Primer A (10 µM) 2.1 µl Primer B (10 µM) 2.1 µl Rehydration Buffer 29.5 µl Template and dH2O 1.2 and 12.6 µl Total Volume 47.5 µl
2	Transfer 47.5 µl of the rehydration solution to the reaction pellet and mix.
3	Add 2.5 µl 280 mM magnesium acetate and mix.
4	Incubate at 38 °C for 30 min. After 4min of incubation mix the reaction by flicking the tube
5	Detect the amplicon using LF strips or 2 % agarose gel electrophoresis.

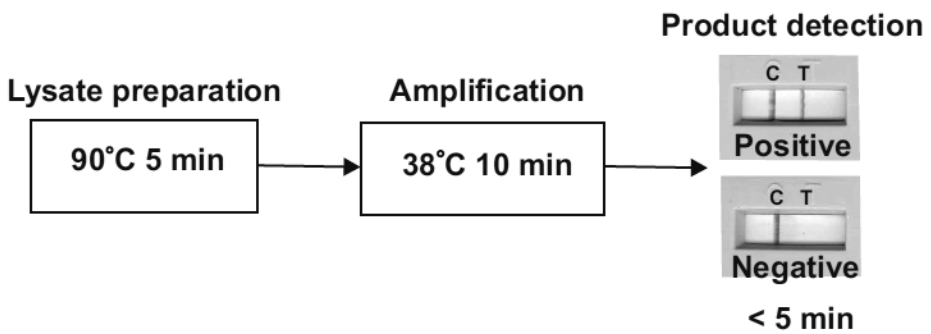


Figure 4. *Chlamydia trachomatis* detection assay. The entire amplification process takes less than 20 min and requires incubation of urine samples at 90 °C for 5 min for sample preparation, then 5 µl of heated urine sample are applied on CT RPA assay at 38 °C for 10 min for product amplification. The amplified products are then detected by lateral flow dipsticks.

3.3 Primer design

We designed the RPA primers according to the manufacturer's guidelines and tested all of these primers to avoid unspecific amplification or primer dimer formation. The best primer pair was found to be biotin/FAM labeled to be detectable on the lateral-flow strips. These are presented in **Table 4**.

Table 4. *C. trachomatis* primer sequences for RPA assay

Target organism and region	Amplification product size	primer name	5' modification	Sequence
C. trachomatis CDS2 (cryptic plasmid)	116 bp	CDS2-FW	Biotin	5' – CCT TCA TTA TGT CGG AGT CTG AGC ACC CTA GGC - 3'
		CDS2-RV	FAM	5' - CTC TCA AGC AGG ACT ACA AGC TGC AAT CCC TT- 3'

We designed LAMP primers with LAMP Designer 1.10 software (PREMIER Biosoft) and using Primer-BLAST (NCBI). The best designed primer sets are listed in **Table 5**. We avoided oligonucleotides that contain sequence elements that promote secondary structures and primer-primer interactions or hairpins when selecting primers for LAMP/RPA.

Table 5. LAMP primer set selected for C. trachomatis specific LAMP. This primer set recognizes the CDS2 gene region on the plasmid target.

Name	Sequence
F3	5' AAT ATC ATC TTT GCG GTT GC 3'
B3	5' TCT ACA AGA GTA CAT CGG TCA 3'
FIP	5' Biotin-TCG AGC AAC CGC TGT GAC GAC CTT CAT TAT GTC GGA GTC 3'
BIP	5' FAM-GCA GCT TGT AGT CCT GCT TGA GTC TTC GTA ACT CGC TCC 3'
LF	5' Biotin-TAC AAA CGC CTA GGG TGC 3'
LB	5' FAM-CGG GCG ATT TGC CTT AAC 3'

3.4 DNA standards for LAMP/RPA assay sensitivity/specificity determination

To determine the sensitivity of the RPA/LAMP assay, a *C. trachomatis* CDS2 gene fragment was cloned into a pGL3-Promoter vector (Promega, Madison, WI). The resulting pGL3-CDS2 plasmid was used as a quantitative molecular standard.

We determined the sensitivity of the analytical assay using different dilution series of BglII linearised pGL3-CDS2 plasmid template (Krolov et al., 2014). In parallel, we also analyzed the assay sensitivity using a *C. trachomatis* strain UW-36/Cx genomic DNA as total DNA extracted from infected HeLa cells (ATCC VR-886D). We extracted the total DNA from clinical samples using a QIAamp Viral RNA Mini Kit (Qiagen).

3.5 Sample pre-treatment

In the RPA assay we used a sample heat-pretreatment method for direct *Chlamydia trachomatis* detection (90 °C 5 min). Lysates used in the LAMP reaction were also heated at 90 °C or treated with an antimicrobial peptide lysis mix (SelfD Technology GmbH, Leipzig, Germany; commercial component). We used this approach to evaluate the sensitivity of the *C. trachomatis* RPA/LAMP based assay performed directly on biological samples without DNA purification.

3.6 Statistical analysis

The sensitivity, specificity and 95% confidence interval (CI) for each assay was calculated using a logistic regression model.

3.7 Detection of the product

The detection of the LAMP *C. trachomatis* amplification product was performed using either lateral flow dipsticks, real-time monitoring, or by gel electrophoresis.

The RPA product was analyzed by gel electrophoresis and lateral flow dipsticks.

3.8 Clinical specimen collection and storage

To evaluate the analytical sensitivity of our LAMP/RPA assay, we obtained first-void morning urine samples from the Sexual Health Clinique (Tartu, Estonia) for *C. trachomatis* analysis. To evaluate the sensitivity and specificity of the RPA and LAMP assays, first void urine samples were collected from 70 and 650 patients, respectively, who were attending the sexual health clinic in

April and May 2013 (Tartu, Estonia) and in October 2013 to December 2014. Patients between the ages of 18 to 25 years old were directed by the clinician to test for sexually transmitted diseases, and those who volunteered to participate in our study donated self-collected morning first-void urine samples. The Research Ethics Committee of the University of Tartu approved this study. The criteria for patient enrolment in this study were: change of sexual partner, un-protected sexual intercourse, sexually transmitted infection of partner, multiple sexual partners, symptoms of sexually transmitted disease. Once received, urine samples were tested by the *C. trachomatis* specific LAMP/RPA assay within 6 h after collection. We compared our results with laboratory results (Roche Cobas Amplicor *C. trachomatis* assay). We also used CT negative pooled urine (a urine mix from five men and five women in equal volumes) to evaluate the assay sensitivity.

4. RESULTS AND DISCUSSION

4.1 Recombinase polymerase amplification based assay (RPA) enables highly sensitive and specific pathogen detection directly from unprocessed urine sample (Paper I)

The focus of the research presented here was to develop and evaluate a recombinase polymerase amplification assay to detect *Chlamydia trachomatis* that can be successfully performed on pure DNA as well as minimally processed urine samples without significant loss of sensitivity.

RPA technology was introduced in 2006 by Piepenburg and coworkers and has since been commercialized for research use only (TwistDx, Inc.) (Piepenburg et al., 2006). The reaction progresses rapidly and results in specific DNA amplification within 10–30 min without thermocycling. The entire reaction system is contained in a stable, lyophilized formulation and can be transported safely without refrigeration. Thus, providing a good basis for a POC diagnostic platform.

First, we identified conserved CDS2 regions on the cryptic plasmid of *Chlamydia trachomatis* and designed RPA primers that target these regions for specific *C. trachomatis* detection. The cryptic plasmid is a preferred target for *Chlamydia trachomatis* detection due to its multiplicity, which is normally between 4–10 copies per cell (Pickett et al., 2005). Besides, the plasmid is useful as a target for diagnostic testing, because it is relatively stable and hence more resistant to nuclease damage than the genome. This, in turn, can significantly increase sensitivity of diagnostics assay. We selected a primer pair that were highly sensitive and specific for *C. trachomatis* detection and did not produce any background signal on the lateral-flow strips. We found that our newly developed CT RPA is a very promising method for point-of-care application. As low as 50 copies of pGL3_CDS2 plasmid or 0.2 pg of *C. trachomatis* genomic DNA were amplified and detected within 20 min at 38 °C in a one-step procedure, which corresponds to 5–12 pathogens per reaction (Figure 1A in Ref. I). At the same time, the amplification product can be easily detected using the lateral-flow dipsticks, which can also be easily integrated in a POC test thereby enabling reaction product detection within 10 min. A layout of this assay is provided in **Figure 5.**

We showed that this CT CDS2 specific RPA assay specifically detects *Chlamydia trachomatis* and is not affected by the presence of excess amounts of human or other bacterial DNA agents such as *Mycoplasma genitalium, Neisseria gonorrhoeae, Ureaplasma urealyticum,* and *Escherichia coli* (Figure 1 in Ref. I). We also succeeded in detecting *Chlamydia trachomatis* directly from heat-treated *Chlamydia trachomatis* positive urine samples (10% of final urine concentration in the reaction) whereas no amplification signal was observed in *Chlamydia trachomatis* negative samples. Thus, RPA does not require a DNA purification step before the amplification reaction. This makes the entire assay

less labor-intensive and time-consuming. At the same time, we noticed that 10% urine in the final assay can slightly inhibit the amplification efficiency and weaken the amplification signal on the lateral flow dipsticks (Figure S2 in Ref. I). This could be due to the complex nature of urine (Cahillet al., 2003). At the same time we found that between 0.05 to 5 ul of urine alone contain a sufficient amount of pathogen DNA to form a positive signal. These results indicate that this *Chlamydia trachomatis* specific RPA assay does not require DNA purification prior to amplification with 5 min heating at 90 °C, which is sufficient to release DNA from the cells and successfully detect *C. trachomatis*. Despite this, there is a clear need to overcome the urine inhibition effect by further optimizing the RPA reaction and/or sample collection procedure.

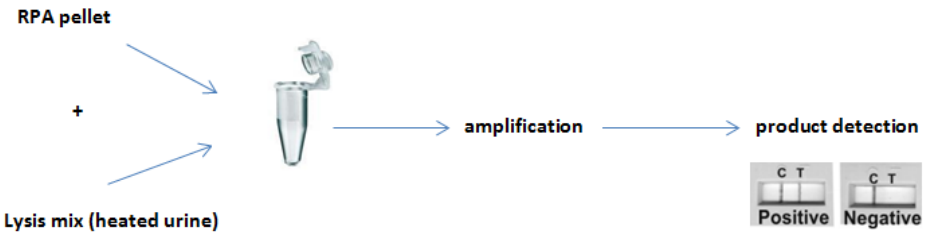


Figure 5. Layout of *Chlamydia trachomatis* detection assay. 5 µl of heated urine are mix with RPA pellet and incubate at 38 °C, the product can be then analyzed by lateral flow dipsticks.

Next, we evaluated the applicability and sensitivity of the *C. trachomatis* specific RPA assay directly on clinical samples. We estimated the sensitivity of our CT RPA assay using 70 self-collected first-void morning urine samples from young adults and compared them with the results of Roche Cobar Amplicor *C. trachomatis* assay carried out in the Tartu University Hospital United Laboratories. For that, all urine samples from the clinical study were briefly incubated at 90 °C for 5 min and then tested using our *C. trachomatis* RPA assay. Of the 12 *C. trachomatis* patients, ten tested positive using our RPA assay when 5 µl of first void urine was heated at 90 °C and used as a template. Based on this, our assay showed 83% (95% CI, 51–97%) sensitivity and 100% (95% CI, 92–100%) specificity (Table 2 in Ref. I). This can be due to the limited amount of added urine sample in final assay because a higher amount (more than 10%) can significantly reduce the sensitivity of the RPA assay. We can also conclude that urine components affect the sensitivity of this RPA assay because when we used purified total DNA from these urine samples, all 12 *C. trachomatis* positive samples tested positive in the RPA assay. The sensitivity of this assay could be further improved by optimizing sample collection, applying an enrichment method, or eliminating crude sample inhibitors.

Currently, no *C. trachomatis* point-of-care test is able to offer high sensitivity or at least a sensitivity comparable with NAATs. We also succeeded in demonstrating that the RPA assay we developed provides highly specific and

sensitive *C. trachomatis* detection when compared with currently available *C. trachomatis* POC assays. Several studies have shown very poor pathogen sensitivities for POC tests (up to 40%), which hinder their large-scale application (Sabido et al., 2009; van der Helm et al., 2012; van Dommelen et al., 2010). However, our novel assay for *C. trachomatis* detection serves a good platform for pathogen detection directly from minimally processed samples due to its on-site application, short time-to-result, and simplicity. In addition, this assay does not require any expensive thermocycling machinery and therefore can be easily applied in point-of-care settings.

4.2 Antimicrobial peptide lysis mix can be efficiently applied on loop mediated isothermal amplification (LAMP) for direct detection of pathogen from biological sample (Paper II)

In this study, we used LAMP-based methods to detect *Chlamydia trachomatis* in combination with antimicrobial peptides for efficient pathogen lysis prior to nucleic acid amplification.

Loop mediated isothermal DNA amplification is a powerful novel and innovative gene amplification technique that is gaining attention as a simple rapid diagnostic tool for the early detection and identification of infectious diseases (Dhama et al., 2014). The advantages of the LAMP method can be applied on various sample materials because it is less prone to inhibitory factors than the classical PCR method [40]. This technique allows accurate and robust amplification of the target sequence of various infectious diseases in a single heating step (60–65 °C) performed in a hot-water bath followed by visualization with a lateral-flow dipstick.

As mentioned above, the current gold standard methods for *C. trachomatis* detection are widely used PCR-based techniques which are both sensitive and specific, yet are only suitable for use in centralized hospital facilities and require both complicated and expensive laboratory apparatus and extraction and concentration of the pathogen genetic material from the sample prior to amplification. Loop mediated amplification methods can provide a good alternative to PCR-based diagnostics in laboratories with low or limited resources and point of care settings. The main advantages of POC tests include rapid availability of data, decreased turnaround time, immediate or almost immediate decision making, rapid response to critical values, easily portable, and decreased patient costs (Fiallos et al., 2001). Several studies have found that up to 50% of tested patients never return to obtain the diagnosis results and are thereby left untreated (Schwebkeet al., 1997). However, the LAMP method could provide a sensitive, specific, and cost effective *C.trachomatis* diagnostic platform.

To begin, we designed specific primers to detect a CDS2 sequence on the cryptic plasmid of *C. trachomatis*. To reduce the time needed to both detect the

pathogen and increase the sensitivity, we designed additional loop primers (LF/LB). These primers display high sensitivity and were able to detect as few as 25 plasmid copies in buffer, however, their sensitivity diminished to 75 plasmid copies when 10% urine was present in the amplification reaction (Table 1 in Ref.II). This clearly demonstrates that urine can diminish the sensitivity of the assay as in our CT RPA assay, where the presence of 10% of urine weakened the amplification signal. The time required to obtain a detectable result was estimated to be 21 min. The resulting assay was 100% specific and did not produce any background signal with 30 pathogens commonly found in urine samples. The amplification product is easily visualized using lateral-flow dipsticks or intercalating fluorescent dyes such as EvaGreen.

Our goal was to design an accurate POC nucleic acid test to detect *C. trachomatis* directly from urine samples without purifying the genomic DNA. We established that 10% urine is sufficient for pathogen detection, thereby making this assay very attractive for pathogen detection directly from unprocessed samples. To the best of our knowledge, this is the first study that evaluates if treating urine samples with an antimicrobial peptide can eliminate the need for DNA isolation procedures. Antimicrobial peptides have previously been shown to lyse different Gram-positive/negative bacteria cells (Carriel-Gomes et al., 2007; Suttmann et al., 2008), thereby releasing DNA from the sample. We hypothesized that this could be a useful biological sample preparation method prior to nucleic acid amplification. Previously, we described that heat biological samples can also aid in the release of pathogenic DNA from cells. We estimate that adding up to 1,2 % antimicrobial peptide mix does not inhibit the efficiency of LAMP amplification (Figure 2 in Ref. II) and is able to successfully lyse *C. trachomatis* EBs (data not shown).

In addition, we evaluated the prevalence of asymptomatic *C. trachomatis* cases from a clinical study (N=650). Approval for that study was obtained from the Research Ethics Committee of the University of Tartu. Based on clinical data, 157 of 650 patients (24%) had STI symptoms whereas 493 patients (76%) had no symptoms. This supports previous findings that *C. trachomatis* infection is mainly asymptomatic (Molano et al., 2005; Morre et al., 2002) and shows the importance of rapid diagnostics of this pathogen for early treatment of the disease.

We evaluated our newly developed *C. trachomatis* specific LAMP assay in a clinical study involving 91 *C. trachomatis* positive patients by comparing our analysis of their urine samples with Roche Cobas CT assay results. The results from this clinical study demonstrate that LAMP, together with a peptide/ detergent lysis mix provides the highest assay sensitivity 73% (95% CI, 39,03–93,98%) as compared with heat treated urine samples 64% (95% CI, 30,79–89,07%). The assay was 100% specific (95% CI, 95,5–100%) (Table 2 in Ref. II). These data clearly show that pre-treatment of urine with a peptide/ lysis detergent provides the LAMP based assay better access to the pathogenic DNA than treatment with heat (73% vs 64%); although this method was not as sensitive as heat treatment in an RPA assay (73% vs 83%).

However, RPA shows higher sensitivity when compared with a LAMP CT assay (Krõlov et al., 2014), although both display a specificity of 100%. Both of these methods could be successfully applied in POC diagnostics because they both display higher sensitivity compared with available CT POC tests that show alarmingly poor pathogen detection sensitivities between 10 to 40 % (Hurley et al., 2014; van der Helm et al., 2012; Nunez-Forero et al., 2016; Van Dommelen et al., 2010). However, in comparison with commercially available nucleic acid based techniques, our developed CT RPA/LAMP assays displays lower sensitivity (73–83% vs 90–99%). However, further optimizations of the LAMP/RPA assays, such as sample collection, concentration, and specific treatment could increase our assay sensitivity to levels comparable with diagnostic assays used in laboratories. Regardless of this, our newly developed *C. trachomatis* specific LAMP assay shows for the first time that a simple and advantageous addition of an antimicrobial peptide mixture aids in the direct detection of pathogens from crude biological samples. Our LAMP assay does not require prior extraction or purification of the genetic material from the pathogen and, as with the RPA assay, can also be integrated into a POC diagnostic platform.

4.3 Improvement of detection of *Chlamydia trachomatis* through the application of different polymerases with a strand displacement activity and evaluation of main urine inhibitors on their activity using LAMP assay (Paper III)

Detection of different sexually transmitted pathogens is mainly accomplished using two strategies for amplifying a defined sequence of nucleic acid: molecular methods such as PCR, and isothermal amplification methods such as LAMP, RPA, SDA, and RCA. PCR is a common and often indispensable technique used in medical and biological research labs for a variety of applications, however, it relies upon instrument based thermal cycling and thermostable DNA polymerase. Although methods based on amplification can greatly reduce the detection time compared with conventional culture-based methods, those rapid amplification methods are limited by the number of pathogens present in the sample or by the inhibition of many components found in biological samples. Extraneous compounds can degrade or even compete with DNA itself, thereby reducing the assay sensitivity and/or specificity (Chernesky et al., 1997).

Because DNA polymerases are the key enzymes that perform these reactions and influence the sensitivity and robustness of all molecular assays, a good strategy to improve assay sensitivity and performance time is to find inhibitor resistant DNA polymerases. Enzymes used in PCR (e.g., Taq DNA polymerase) possess high thermostability and robust polymerase activity, however, they typically lack efficient strand displacement activity, which makes them unsuitable for isothermal amplification methods such as LAMP. LAMP was first

developed in the year 2000 by Notomi and colleagues (Notomi et al., 2000) and has since been shown to exhibit high analytical sensitivity, specificity and tolerance to various inhibitors. Originally, the LAMP method used Bst DNA polymerase with strand displacement activity, however, since then various polymerases and their derivates with a variety of properties have been developed to perform more efficient isothermal amplification. Despite this, most reports continue to use LAMP for the detection of already extracted and purified DNA from crude samples. It is thus advantageous to search for a polymerase that is both rapid and tolerates the main inhibitors present in crude samples to avoid the long and tedious genomic DNA purification steps prior to amplification.

For that reason, we tested the efficiency of the following DNA polymerases for LAMP amplification that all possess strand displacement activity: Bsm, Bst, Bst2.0, Bst2.0WarmStart, GspSSD, GspSSD2.0, GspM, GspM2.0, Tin, OmniAmp, and SD in the presence of urine and/or the main inhibitors typically found in urine samples at physiologically relevant concentrations.

First, we evaluated the efficiency of all 11 polymerases in a model system and compared them with the Bsm polymerase that we used in the CT LAMP assay. We previously evaluated that the optimum amplification time for the Bsm polymerase is 21 min. We found that the fastest polymerase GspSSD2.0 (amplification after 10 min) always produced an unspecific background signal after 30 min of amplification even in the absence of template DNA. Polymerases such as Bst, Bst2.0, Bst2.0WarmStart, GspSSD, GspSSD2.0, GspM2.0, and OmniAmp also display good amplification and all were able to produce an amplification signal already after 25 min. The worst polymerases in terms of speed were SD and Tin, which were significantly slower than Bsm (Supplemental Figure1 in Ref. III).

Next, we evaluated the urine tolerance of these 11 polymerases. The results revealed that best polymerases in terms of urine tolerance are Bsm and GspM2.0 (up to 20%). However, polymerases such as GspM, Bst, Bst2.0, Bst2.0WarmStart, and GspSSD2.0 can also tolerate up to 15% urine without significantly affecting the amplification time. OmniAmp, Tin, and SD polymerases were the most sensitive to the presence of urine and are thus not suitable for direct detection of pathogenic DNA from urine samples (Figure 1 in Ref III).

We found that the presence of the main urine components such as BSA, Mg^{2+}, acidic or alkaline conditions, or urea at physiologically relevant concentrations has no impact on the activity of the polymerases we tested (Supplemental Figure 3, 4, 5 in Ref. III). However, the addition of NaCl significantly affected the activity of all polymerases.

We found that both the Bsm and GspM2.0 polymerases had the highest level of salt tolerance (up to 45 mM NaCl) without any reduction in amplification efficiency, which correlates well with high urine tolerance of these polymerases. Polymerases such as GspSSD, GspSSD2.0, Bst2.0, Bst2.0WarmStart, and GspM are able to tolerate up to 15 mM NaCl (a physiologically relevant NaCl concentration) without significant prolongation of amplification time. Polymerases such as SD, OmniAmp, and Tin are very sensitive to the presence of

salt and are therefore less applicable for the direct detection of pathogens in urine (Figure 2 in Ref. III).

These findings could form the basis for the development of an improved nucleic acid based isothermal amplification method for direct nucleic acid detection from biological samples, simplifying the whole nucleic acid detection assay, and allowing it to be applicable for POC assays which aim to reduce the prevalence of undiagnosed infections by providing rapid and efficient diagnosis.

5. CONCLUSIONS

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

- We devised a rapid and highly sensitive detection assay of *C. trachomatis* directly from heat-treated urine samples using a recombinase polymerase amplification method. This assay was able to detect as few as 5 pathogens per reaction within 20 min and does not require DNA purification prior to amplification. The simplicity, sensitivity, low cost, and speed of this *C. trachomatis* RPA assay enable its application in POC settings.

- We combined a *Chlamydia trachomatis* loop-mediated isothermal amplification assay with antimicrobial peptide lysis for the direct detection of *C. trachomatis* from urine samples. The assay requires 21 min and does not include DNA extraction and purification steps. The final product can be detected using lateral flow strips and is therefore applicable in point-of-care settings.

- We evaluated the efficiency of polymerases with strand displacement activity in the presence of the main urine inhibitors in an attempt to find a more rapid and urine tolerant polymerase. By monitoring the LAMP reaction in real-time, we found that the most critical component in urine that can reduce assay sensitivity is salt. We found that polymerases such as Bsm and GspM2.0 have the highest level of salt tolerance and are able to function in the presence of up to 45 mM NaCl without any reduction in amplification activity and up to 20% or 10%, urine respectively.

In conclusion, the research presented in this dissertation found that both the RPA and LAMP isothermal amplification methods could be successfully applied on pure DNA as well as minimally processed urine samples and can provide a good basis for developing a point-of-care diagnostics platform. We also found that salt is one of the most crucial components in urine and affects polymerases that possess strand displacement activity. In addition, we found that some polymerases, such as Bsm or GspM2.0, maintained their amplification activity in the presence of NaCl at physiologically relevant concentrations and are therefore applicable for the direct detection of pathogenic DNA in urine. These findings can aid in the development of improved nucleic acid based isothermal amplification methods for direct pathogen detection from biological samples.

SUMMARY IN ESTONIAN

Sugulisel teel leviva bakteri *Chlamydia trachomatise* tuvastamine isotermiliste amplifikatsiooni meetodite abil

Praeguseks on teada enam kui 30 erinevat sugulisel teel levivat bakterit, viirust ja parasiiti. Nendega nakatamisel on enimesinevateks sümptomiteks: suurenenud limasmädane voolus; krooniline alakõhuvalu; valulik urineerimine; veritsused jne. Õigeaegse ravi puudumine võib oluliselt kahjustada reproduktiivorganeid, tekitades põletikku emakakaelas, munajuhas või kusitis, lisaks ka suureneb emakaväliseraseduse ja isegi viljatuse tõenäosus. Nende patogeenide tõelise esinemissageduse määramist raskendab haiguse aladiagnoos, sest valdavalt kulgevad/võivad kulgeda suguhaigused asümptomaatiliselt. Seetõttu on haigusetekitajate kiire ja täpne tuvastamine infektsiooni varases staadiumis oluline, et alustada kohe õige raviga ning ennetada tõsisemate tagajärgede teket.

Antud projekti peamiseks eesmärgiks oli arendada ja optimeerida kiire, täpne ning usaldusväärne isotermilise amplifikatsiooni meetod sugulisel teel leviva bakteri *C. trachomatise* tuvastamiseks otse uriinist, mida oleks võimalik tulevikus rakendada kodukasutuseks mõeldud kiirtestides.

Selleks võtsime kasutusele kaks isotermilist amplifikatsiooni meetodit: LAMP ja RPA. Nende meetodite põhilisteks eelisteks on kiirus, täpsus, odavus ning tundlikus. Need meetodid vajavad lihtsa aparatuuri ning neid on võimalik tulevikus rakendada kiirtestides.

Esimese töö eesmärgiks oli uurida optimeeritud RPA meetod tundlikkust ja spetsiifilisust *Chlamydia trachomatise* otseseks tuvastamiseks uriinist. Tulemusena selgus, et vastava meetodiga oli küll võimalik tuvastada patogeeni otse uriinist, kuid vaatamata 100% spetsiifilisusele oli tundlikkus vaid 83% .

Teises töös uuriti LAMP meetodi tundlikkust *C. trachomatise* detekteerimiseks. Kui RPA puhul oli eelnevalt tehtud uriini eeltöötlus kuumutamise abil (90 °C juures 5 minutit), siis nüüd kasutati proovi eeltöötlemiseks lüüsisegu, mis sisaldas antimikroobset peptiidi. Kliiniline uuring näitas, et sellise meetodi spetsiifilisus oli 100% ja tundlikkus 73% .

Kolmandas töös uuriti kui tundlikud on erinevad polümeraasid nii uriini kui ka erinevate uriini komponentide suhtes. Eesmärgiks oli leida polümeraas, mis oleks võimalikult vähetundlik uriini komponentide olemasolule ning mida saaks rakendada patogeeni otseseks detekteerimiseks uriinist. Selleks, et hinnata uriini komponentide mõju nendele polümeraasideleanalüüsisime 11 erinevat polümeraasi LAMP isotermilises amplifikatsioonis. See võimaldas leida polümeraase, mis on kõrge uriini taluvusega, mis omakorda lihtsustaks patogeeni tuvastamist, kuna võimaldab vältida DNA puhastamise etapi bioloogilisest materjalist. Selle uurimise käigus selgus, et põhiline uriini komponent, mis mõjutab kõikide polümeraaside aktiivsust on uriinis sisalduv sool. Teised komponendid nagu uurea, seerum albumiin, magneesium, aluseline/happeline keskkond ei mõjutanud uuritavate polümeraaside aktiivsust.

Kokkuvõtteks, nii RPA kui ka LAMP meetodeid on võimalik rakendada kiirtestidena *Chlamydia trachomatise* otseseks detektsiooniks uriinis. Siiski, et tõsta nende meetodite tundlikkust, võiks mõelda nende edasisele optimeerimisele nagu uriini kontsentreerimine, eeltöötluse optimeerimine ning polümeraasi vahetamine, mis lubaks tõsta diagnostilise meetodi tundlikkust.

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REFERENCES

Abd El Wahed, A., Patel, P., Faye, O., Thaloengsok, S., Heidenreich, D., Matangkasombut, P., . . . Weidmann, M. (2015). Recombinase Polymerase Amplification Assay for Rapid Diagnostics of Dengue Infection. *PLoS One, 10*(6), e0129682. doi: 10.1371/journal.pone.0129682

Abdelrahman, Y. M., & Belland, R. J. (2005). The chlamydial developmental cycle. *FEMS Microbiol Rev, 29*(5), 949–959. doi: 10.1016/j.femsre.2005.03.002

Abu Al-Soud, W., & Radstrom, P. (1998). Capacity of nine thermostable DNA polymerases To mediate DNA amplification in the presence of PCR-inhibiting samples. *Appl Environ Microbiol, 64*(10), 3748–3753.

Abu Al-Soud, W., & Radstrom, P. (2000). Effects of amplification facilitators on diagnostic PCR in the presence of blood, feces, and meat. *J Clin Microbiol, 38*(12), 4463–4470.

Akane, A., Matsubara, K., Nakamura, H., Takahashi, S., & Kimura, K. (1994). Identification of the heme compound copurified with deoxyribonucleic acid (DNA) from bloodstains, a major inhibitor of polymerase chain reaction (PCR) amplification. *J Forensic Sci, 39*(2), 362–372.

Al-Soud, W. A., & Radstrom, P. (2001). Purification and characterization of PCR-inhibitory components in blood cells. *J Clin Microbiol, 39*(2), 485–493. doi: 10.1128/JCM.39.2.485-493.2001

Albrecht, M., Sharma, C. M., Reinhardt, R., Vogel, J., & Rudel, T. (2010). Deep sequencing-based discovery of the Chlamydia trachomatis transcriptome. *Nucleic Acids Research, 38*(3), 868–877. doi: 10.1093/nar/gkp1032

Ali, M. M., Li, F., Zhang, Z., Zhang, K., Kang, D. K., Ankrum, J. A., . . . Zhao, W. (2014). Rolling circle amplification: a versatile tool for chemical biology, materials science and medicine. *Chem Soc Rev, 43*(10), 3324–3341. doi: 10.1039/c3cs60439j

Apari, P., de Sousa, J. D., & Muller, V. (2014). Why sexually transmitted infections tend to cause infertility: an evolutionary hypothesis. *PLoS Pathog, 10*(8), e1004111. doi: 10.1371/journal.ppat.1004111

Arnold, S. R., & Ford-Jones, E. L. (2000). Congenital syphilis: A guide to diagnosis and management. *Paediatr Child Health, 5*(8), 463–469.

Belland, R. J., Zhong, G., Crane, D. D., Hogan, D., Sturdevant, D., Sharma, J., . . . Caldwell, H. D. (2003). Genomic transcriptional profiling of the developmental cycle of Chlamydia trachomatis. *Proc Natl Acad Sci U S A, 100*(14), 8478–8483. doi: 10.1073/pnas.1331135100

Betsou, F., Beaumont, K., Sueur, J. M., & Orfila, J. (2003). Construction and evaluation of internal control DNA for PCR amplification of Chlamydia trachomatis DNA from urine samples. *J Clin Microbiol, 41*(3), 1274–1276.

Black, C. M. (1997). Current methods of laboratory diagnosis of Chlamydia trachomatis infections. *Clin Microbiol Rev, 10*(1), 160–184.

Brunelle, B. W., & Sensabaugh, G. F. (2006). The ompA gene in Chlamydia trachomatis differs in phylogeny and rate of evolution from other regions of the genome. *Infect Immun, 74*(1), 578–585. doi: 10.1128/IAI.74.1.578-585.2006

Cahill, D. J., Fry, C. H., & Foxall, P. J. (2003). Variation in urine composition in the human urinary tract: evidence of urothelial function in situ? *J Urol, 169*(3), 871–874. doi: 10.1097/01.ju.0000052404.42651.55

Carriel-Gomes, M. C., Kratz, J. M., Barracco, M. A., Bachere, E., Barardi, C. R., & Simoes, C. M. (2007). In vitro antiviral activity of antimicrobial peptides against

herpes simplex virus 1, adenovirus, and rotavirus. *Mem Inst Oswaldo Cruz, 102*(4), 469–472.

Centers for Disease, C., & Prevention. (2014). Recommendations for the laboratory-based detection of Chlamydia trachomatis and Neisseria gonorrhoeae – 2014. *MMWR Recomm Rep, 63*(RR-02), 1–19.

Chan, E. L., Brandt, K., & Horsman, G. B. (1994). A 1-year evaluation of Syva Micro-Trak Chlamydia enzyme immunoassay with selective confirmation by direct fluorescent-antibody assay in a high-volume laboratory. *J Clin Microbiol, 32*(9), 2208–2211.

Chen, X., Wang, B., Yang, W., Kong, F., Li, C., Sun, Z., . . . Gilbert, G. L. (2014). Rolling circle amplification for direct detection of rpoB gene mutations in Mycobacterium tuberculosis isolates from clinical specimens. *J Clin Microbiol, 52*(5), 1540–1548. doi: 10.1128/JCM.00065-14

Chernesky, M. A. (2005). The laboratory diagnosis of Chlamydia trachomatis infections. *Can J Infect Dis Med Microbiol, 16*(1), 39–44.

Chernesky, M. A., Jang, D., Sellors, J., Luinstra, K., Chong, S., Castriciano, S., & Mahony, J. B. (1997). Urinary inhibitors of polymerase chain reaction and ligase chain reaction and testing of multiple specimens may contribute to lower assay sensitivities for diagnosing Chlamydia trachomatis infected women. *Mol Cell Probes, 11*(4), 243–249. doi: 10.1006/mcpr.1997.0109

Chernesky, M. A., Mahony, J. B., Castriciano, S., Mores, M., Stewart, I. O., Landis, S. J., . . . Leman, C. (1986). Detection of Chlamydia trachomatis antigens by enzyme immunoassay and immunofluorescence in genital specimens from symptomatic and asymptomatic men and women. *J Infect Dis, 154*(1), 141–148.

Choopara I &Somboonna N. Development of Chlamydia trachomatis detection by loop-mediated isothermal amplification. Second International Conference on Advances in Applied Science and Environmental Engineering – ASEE 2014

Choopara, I., Arunrut, N., Kiatpathomchai, W., Dean, D., & Somboonna, N. (2017). Rapid and visual Chlamydia trachomatis detection using loop-mediated isothermal amplification and hydroxynaphthol blue. Lett Appl Microbiol, 64(1), 51–56. doi: 10.1111/lam.12675

Cohen, M. S., Hoffman, I. F., Royce, R. A., Kazembe, P., Dyer, J. R., Daly, C. C., . . . Eron, J. J., Jr. (1997). Reduction of concentration of HIV-1 in semen after treatment of urethritis: implications for prevention of sexual transmission of HIV-1. AIDSCAP Malawi Research Group. *Lancet, 349*(9069), 1868–1873.

Connelly, J. T., Nugen, S. R., Borejsza-Wysocki, W., Durst, R. A., Montagna, R. A., & Baeumner, A. J. (2008). Human pathogenic Cryptosporidium species bioanalytical detection method with single oocyst detection capability. *Anal Bioanal Chem, 391*(2), 487–495. doi: 10.1007/s00216-008-1967-2

Cosentino, L. A., Landers, D. V., & Hillier, S. L. (2003). Detection of Chlamydia trachomatis and Neisseria gonorrhoeae by strand displacement amplification and relevance of the amplification control for use with vaginal swab specimens. *J Clin Microbiol, 41*(8), 3592–3596.

Crotchfelt, K. A., Pare, B., Gaydos, C., & Quinn, T. C. (1998). Detection of Chlamydia trachomatis by the Gen-Probe AMPLIFIED Chlamydia Trachomatis Assay (AMP CT) in urine specimens from men and women and endocervical specimens from women. *J Clin Microbiol, 36*(2), 391–394.

Deiman, B., van Aarle, P., & Sillekens, P. (2002). Characteristics and applications of nucleic acid sequence-based amplification (NASBA). *Mol Biotechnol, 20*(2), 163–179. doi: 10.1385/MB:20:2:163

Dhama, K., Karthik, K., Chakraborty, S., Tiwari, R., Kapoor, S., Kumar, A., & Thomas, P. (2014). Loop-mediated isothermal amplification of DNA (LAMP): a new diagnostic tool lights the world of diagnosis of animal and human pathogens: a review. *Pak J Biol Sci, 17*(2), 151–166.

Doseeva, V., Forbes, T., Wolff, J., Khripin, Y., O'Neil, D., Rothmann, T., & Nazarenko, I. (2011). Multiplex isothermal helicase-dependent amplification assay for detection of Chlamydia trachomatis and Neisseria gonorrhoeae. *Diagn Microbiol Infect Dis, 71*(4), 354–365. doi: 10.1016/j.diagmicrobio.2011.08.021

Eboigbodin, K. E., & Hoser, M. J. (2016). Multiplex Strand Invasion Based Amplification (mSIBA) assay for detection of Chlamydia trachomatis and Neisseria gonorrhoeae. *Sci Rep, 6*, 20487. doi: 10.1038/srep20487

Edwards, T., Burke, P. A., Smalley, H. B., Gillies, L., & Hobbs, G. (2014). Loop-mediated isothermal amplification test for detection of Neisseria gonorrhoeae in urine samples and tolerance of the assay to the presence of urea. *J Clin Microbiol, 52*(6), 2163–2165. doi: 10.1128/JCM.00314–14

Euler, M., Wang, Y., Heidenreich, D., Patel, P., Strohmeier, O., Hakenberg, S., . . . Weidmann, M. (2013). Development of a panel of recombinase polymerase amplification assays for detection of biothreat agents. *J Clin Microbiol, 51*(4), 1110–1117. doi: 10.1128/JCM.02704-12

European Centre for Disease Prevention and Control. Sexually transmitted infections in Europe 2013; Stockholm: ECDC; 2015

European Centre for Disease Prevention and Control. Chlamydia control in Europe: literature review. Stockholm: ECDC; 2014

Farley, T. A., Cohen, D. A., & Elkins, W. (2003). Asymptomatic sexually transmitted diseases: the case for screening. *Prev Med, 36*(4), 502–509.

Fiallos, M. R., Hanhan, U. A., & Orlowski, J. P. (2001). Point-of-care testing. *Pediatr Clin North Am, 48*(3), 589–599.

Fontana, C., Favaro, M., Cicchetti, O., Minelli, S., Pistoia, E. S., & Favalli, C. (2005). Performance of strand displacement amplification assay in the detection of Chlamydia trachomatis and Neisseria gonorrhoeae. *Jpn J Infect Dis, 58*(5), 283–288.

Fraczyk, M., Wozniakowski, G., Kowalczyk, A., Niemczuk, K., & Pejsak, Z. (2016). Development of cross-priming amplification for direct detection of the African Swine Fever Virus, in pig and wild boar blood and sera samples. *Lett Appl Microbiol, 62*(5), 386–391. doi: 10.1111/lam.12569

Francois, P., Tangomo, M., Hibbs, J., Bonetti, E. J., Boehme, C. C., Notomi, T., . . . Schrenzel, J. (2011). Robustness of a loop-mediated isothermal amplification reaction for diagnostic applications. *FEMS Immunol Med Microbiol, 62*(1), 41–48. doi: 10.1111/j.1574-695X.2011.00785.x

Gandelman, O. A., Church, V. L., Moore, C. A., Kiddle, G., Carne, C. A., Parmar, S., . . . Murray, J. A. (2010). Novel bioluminescent quantitative detection of nucleic acid amplification in real-time. *PLoS One, 5*(11), e14155. doi: 10.1371/journal.pone.0014155

Garland, S. M., Tabrizi, S., Hallo, J., & Chen, S. (2000). Assessment of Chlamydia trachomatis prevalence by PCR and LCR in women presenting for termination of pregnancy. *Sex Transm Infect, 76*(3), 173–176.

Ghuysen, J. M., & Goffin, C. (1999). Lack of cell wall peptidoglycan versus penicillin sensitivity: new insights into the chlamydial anomaly. *Antimicrob Agents Chemother, 43*(10), 2339–2344.

Gill, P., & Ghaemi, A. (2008). Nucleic acid isothermal amplification technologies: a review. *Nucleosides Nucleotides Nucleic Acids, 27*(3), 224–243. doi: 10.1080/15257770701845204

Ginocchio, C. C., Chapin, K., Smith, J. S., Aslanzadeh, J., Snook, J., Hill, C. S., & Gaydos, C. A. (2012). Prevalence of Trichomonas vaginalis and coinfection with Chlamydia trachomatis and Neisseria gonorrhoeae in the United States as determined by the Aptima Trichomonas vaginalis nucleic acid amplification assay. *J Clin Microbiol, 50*(8), 2601–2608. doi: 10.1128/JCM.00748-12

Global Burden of Disease Study, C. (2015). Global, regional, and national incidence, prevalence, and years lived with disability for 301 acute and chronic diseases and injuries in 188 countries, 1990–2013: a systematic analysis for the Global Burden of Disease Study 2013. *Lancet, 386*(9995), 743–800. doi: 10.1016/S0140-6736(15)60692-4

Guatelli, J. C., Whitfield, K. M., Kwoh, D. Y., Barringer, K. J., Richman, D. D., & Gingeras, T. R. (1990). Isothermal, in vitro amplification of nucleic acids by a multienzyme reaction modeled after retroviral replication. *Proc Natl Acad Sci U S A, 87*(19), 7797.

Hamidi, S. V., & Ghourchian, H. (2015). Colorimetric monitoring of rolling circle amplification for detection of H5N1 influenza virus using metal indicator. *Biosensors & Bioelectronics, 72*, 121–126. doi: 10.1016/j.bios.2015.04.078

Hammerschlag, M. R., Ajl, S., & Laraque, D. (1999). Inappropriate use of nonculture tests for the detection of Chlamydia trachomatis in suspected victims of child sexual abuse: A continuing problem. *Pediatrics, 104*(5 Pt 1), 1137–1139.

Haugland, S., Thune, T., Fosse, B., Wentzel-Larsen, T., Hjelmevoll, S. O., & Myrmel, H. (2010). Comparing urine samples and cervical swabs for Chlamydia testing in a female population by means of Strand Displacement Assay (SDA). *BMC Womens Health, 10*, 9. doi: 10.1186/1472-6874-10-9

Hill, J., Beriwal, S., Chandra, I., Paul, V. K., Kapil, A., Singh, T., . . . Vats, A. (2008). Loop-mediated isothermal amplification assay for rapid detection of common strains of Escherichia coli. *J Clin Microbiol, 46*(8), 2800–2804. doi: 10.1128/JCM.00152-08

Hsieh, K., Patterson, A. S., Ferguson, B. S., Plaxco, K. W., & Soh, H. T. (2012). Rapid, sensitive, and quantitative detection of pathogenic DNA at the point of care through microfluidic electrochemical quantitative loop-mediated isothermal amplification. *Angew Chem Int Ed Engl, 51*(20), 4896–4900. doi: 10.1002/anie.201109115

Hu, J., & Zhang, C. Y. (2010). Sensitive detection of nucleic acids with rolling circle amplification and surface-enhanced Raman scattering spectroscopy. *Anal Chem, 82*(21), 8991–8997. doi: 10.1021/ac1019599

Hurly. D. S, Buhrer-Skinner, Badman M, S. G et al., (2014). Field evaluation of the CRT and ACON chlamydia point-of-care tests in a tropical, low-resource setting. Sexually Transmitted Infections, vol. 90, no. 3, pp. 179–184, 2014.

Iwamoto, T., Sonobe, T., & Hayashi, K. (2003). Loop-mediated isothermal amplification for direct detection of Mycobacterium tuberculosis complex, M. avium, and M. intracellulare in sputum samples. *J Clin Microbiol, 41*(6), 2616–2622.

Jevtusevskaja, J., Uusna, J., Andresen, L., Krolov, K., Laanpere, M., Grellier, T., . . . Langel, U. (2016). Combination with antimicrobial peptide lyses improves loop-

mediated isothermal amplification based method for Chlamydia trachomatis detection directly in urine sample. *BMC Infect Dis, 16*, 329. doi: 10.1186/s12879-016-1674-0

Kaneko, H., Iida, T., Aoki, K., Ohno, S., & Suzutani, T. (2005). Sensitive and rapid detection of herpes simplex virus and varicella-zoster virus DNA by loop-mediated isothermal amplification. *J Clin Microbiol, 43*(7), 3290–3296. doi: 10.1128/JCM.43.7.3290-3296.2005

Kaneko, H., Kawana, T., Fukushima, E., & Suzutani, T. (2007). Tolerance of loop-mediated isothermal amplification to a culture medium and biological substances. *J Biochem Biophys Methods, 70*(3), 499–501. doi: 10.1016/j.jbbm.2006.08.008

Kellogg, J. A., Seiple, J. W., & Stroll, E. S. (1993). Direct fluorescent-antibody confirmation of chlamydial antigen below the detection threshold of the chlamydiazyme enzyme-linked immunosorbent assay. *J Clin Microbiol, 31*(6), 1646–1647.

Kermekchiev, M. B., Kirilova, L. I., Vail, E. E., & Barnes, W. M. (2009). Mutants of Taq DNA polymerase resistant to PCR inhibitors allow DNA amplification from whole blood and crude soil samples. *Nucleic Acids Research, 37*(5), e40. doi: 10.1093/nar/gkn1055

Khan, G., Kangro, H. O., Coates, P. J., & Heath, R. B. (1991). Inhibitory Effects of Urine on the Polymerase Chain-Reaction for Cytomegalovirus DNA. *Journal of Clinical Pathology, 44*(5), 360–365. doi: Doi 10.1136/Jcp.44.5.360

Kim, H. J., Tong, Y., Tang, W., Quimson, L., Cope, V. A., Pan, X., . . . Yen-Lieberman, B. (2011). A rapid and simple isothermal nucleic acid amplification test for detection of herpes simplex virus types 1 and 2. *J Clin Virol, 50*(1), 26–30. doi: 10.1016/j.jcv.2010.09.006

Kivlehan, F., Mavre, F., Talini, L., Limoges, B., & Marchal, D. (2011). Real-time electrochemical monitoring of isothermal helicase-dependent amplification of nucleic acids. *Analyst, 136*(18), 3635–3642. doi: 10.1039/c1an15289k

Kohda, C., Chiba, N., Shimokoba, K., Mizuno, K., Negoro, T., Nakano, Y., & Tanaka, K. (2014). A simple smart amplification assay for the rapid detection of human cytomegalovirus in the urine of neonates. *J Virol Methods, 208*, 160–165. doi: 10.1016/j.jviromet.2014.07.034

Kreader, C. A. (1996). Relief of amplification inhibition in PCR with bovine serum albumin or T4 gene 32 protein. *Appl Environ Microbiol, 62*(3), 1102–1106.

Krolov, K., Frolova, J., Tudoran, O., Suhorutsenko, J., Lehto, T., Sibul, H., . . . Langel, U. (2014). Sensitive and rapid detection of Chlamydia trachomatis by recombinase polymerase amplification directly from urine samples. *J Mol Diagn, 16*(1), 127–135. doi: 10.1016/j.jmoldx.2013.08.003

Lamhoujeb, S., Charest, H., Fliss, I., Ngazoa, S., & Jean, J. (2009). Real-time molecular beacon NASBA for rapid and sensitive detection of norovirus GII in clinical samples. *Canadian Journal of Microbiology, 55*(12), 1375–1380. doi: 10.1139/W09-105

Lauderdale, T. L., Landers, L., Thorneycroft, I., & Chapin, K. (1999). Comparison of the PACE 2 assay, two amplification assays, and Clearview EIA for detection of Chlamydia trachomatis in female endocervical and urine specimens. *J Clin Microbiol, 37*(7), 2223–2229.

Lees, M. I., Newnan, D. M., & Garland, S. M. (1988). Simplified culture procedure for large-scale screening for Chlamydia trachomatis infections. *J Clin Microbiol, 26*(7), 1428–1430.

Lees, M. I., Newnan, D. M., & Garland, S. M. (1991). Comparison of a DNA probe assay with culture for the detection of Chlamydia trachomatis. *J Med Microbiol, 35*(3), 159–161. doi: 10.1099/00222615-35-3-159

Little, M. C., Andrews, J., Moore, R., Bustos, S., Jones, L., Embres, C., . . . et al. (1999). Strand displacement amplification and homogeneous real-time detection incorporated in a second-generation DNA probe system, BDProbeTecET. *Clin Chem, 45*(6 Pt 1), 777–784.

Mahony, J. B., & Chernesky, M. A. (1985). Effect of swab type and storage temperature on the isolation of Chlamydia trachomatis from clinical specimens. *J Clin Microbiol, 22*(5), 865–867.

Mahony, J. B., Luinstra, K. E., Sellors, J. W., Pickard, L., Chong, S., Jang, D., & Chernesky, M. A. (1994). Role of confirmatory PCRs in determining performance of Chlamydia Amplicor PCR with endocervical specimens from women with a low prevalence of infection. *J Clin Microbiol, 32*(10), 2490–2493.

Mahony, J. B., Song, X., Chong, S., Faught, M., Salonga, T., & Kapala, J. (2001). Evaluation of the NucliSens Basic Kit for detection of Chlamydia trachomatis and Neisseria gonorrhoeae in genital tract specimens using nucleic acid sequence-based amplification of 16S rRNA. *J Clin Microbiol, 39*(4), 1429–1435. doi: 10.1128/JCM.39.4.1429-1435.2001

Modarress, K. J., Cullen, A. P., Jaffurs, W. J., Sr., Troutman, G. L., Mousavi, N., Hubbard, R. A., . . . Lorincz, A. T. (1999). Detection of Chlamydia trachomatis and Neisseria gonorrhoeae in swab specimens by the Hybrid Capture II and PACE 2 nucleic acid probe tests. *Sex Transm Dis, 26*(5), 303–308.

Molano, M., Meijer, C. J., Weiderpass, E., Arslan, A., Posso, H., Franceschi, S., . . . van den Brule, A. J. (2005). The natural course of Chlamydia trachomatis infection in asymptomatic Colombian women: a 5-year follow-up study. *J Infect Dis, 191*(6), 907–916. doi: 10.1086/428287

Moncada, J., Schachter, J., Shafer, M. A., Williams, E., Gourlay, L., Lavin, B., & Bolan, G. (1994). Detection of Chlamydia trachomatis in first catch urine samples from symptomatic and asymptomatic males. *Sex Transm Dis, 21*(1), 8–12.

Moncada, J., Schachter, J., Shipp, M., Bolan, G., & Wilber, J. (1989). Cytobrush in collection of cervical specimens for detection of Chlamydia trachomatis. *J Clin Microbiol, 27*(8), 1863–1866.

Morre, S. A., Sillekens, P., Jacobs, M. V., van Aarle, P., de Blok, S., van Gemen, B., . . . van den Brule, A. J. (1996). RNA amplification by nucleic acid sequence-based amplification with an internal standard enables reliable detection of Chlamydia trachomatis in cervical scrapings and urine samples. *J Clin Microbiol, 34*(12), 3108–3114.

Morre, S. A., Sillekens, P. T., Jacobs, M. V., de Blok, S., Ossewaarde, J. M., van Aarle, P., . . . van den Brule, A. J. (1998). Monitoring of Chlamydia trachomatis infections after antibiotic treatment using RNA detection by nucleic acid sequence based amplification. *Mol Pathol, 51*(3), 149–154.

Morre, S. A., van den Brule, A. J., Rozendaal, L., Boeke, A. J., Voorhorst, F. J., de Blok, S., & Meijer, C. J. (2002). The natural course of asymptomatic Chlamydia trachomatis infections: 45% clearance and no development of clinical PID after one-year follow-up. *Int J STD AIDS, 13 Suppl 2*, 12–18. doi: 10.1258/095646202762226092

Mukhopadhyay, H. K., Amsaveni, S., Matta, S. L., Antony, P. X., Thanislass, J., & Pillai, R. M. (2012). Development and evaluation of loop-mediated isothermal

amplification assay for rapid and sensitive detection of canine parvovirus DNA directly in faecal specimens. *Lett Appl Microbiol, 55*(3), 202–209. doi: 10.1111/j.1472-765X.2012.03284.x

Nadeau, J. G., Pitner, J. B., Linn, C. P., Schram, J. L., Dean, C. H., & Nycz, C. M. (1999). Real-time, sequence-specific detection of nucleic acids during strand displacement amplification. *Anal Biochem, 276*(2), 177–187. doi: 10.1006/abio.1999.4350

Nagamine, K., Hase, T., & Notomi, T. (2002). Accelerated reaction by loop-mediated isothermal amplification using loop primers. *Mol Cell Probes, 16*(3), 223–229.

Nakao, R., Stromdahl, E. Y., Magona, J. W., Faburay, B., Namangala, B., Malele, I., . . . Sugimoto, C. (2010). Development of loop-mediated isothermal amplification (LAMP) assays for rapid detection of Ehrlichia ruminantium. *BMC Microbiol, 10*, 296. doi: 10.1186/1471-2180-10-296

Newman, L., Kamb, M., Hawkes, S., Gomez, G., Say, L., Seuc, A., & Broutet, N. (2013). Global estimates of syphilis in pregnancy and associated adverse outcomes: analysis of multinational antenatal surveillance data. *PLoS Med, 10*(2), e1001396. doi: 10.1371/journal.pmed.1001396

Newman, L., Rowley, J., Vander Hoorn, S., Wijesooriya, N. S., Unemo, M., Low, N., . . . Temmerman, M. (2015). Global Estimates of the Prevalence and Incidence of Four Curable Sexually Transmitted Infections in 2012 Based on Systematic Review and Global Reporting. *PLoS One, 10*(12), e0143304. doi: 10.1371/journal.pone.0143304

Nicholson, T. L., Olinger, L., Chong, K., Schoolnik, G., & Stephens, R. S. (2003). Global stage-specific gene regulation during the developmental cycle of Chlamydia trachomatis. *J Bacteriol, 185*(10), 3179–3189.

Nixon, G. J., Svenstrup, H. F., Donald, C. E., Carder, C., Stephenson, J. M., Morris-Jones, S., . . . Foy, C. A. (2014). A novel approach for evaluating the performance of real time quantitative loop-mediated isothermal amplification-based methods. *Biomol Detect Quantif, 2*, 4–10. doi: 10.1016/j.bdq.2014.11.001

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., & Hase, T. (2000). Loop-mediated isothermal amplification of DNA. *Nucleic Acids Research, 28*(12). doi: ARTN e6310.1093/nar/28.12.e63

Nugen, S. R., Asiello, P. J., Connelly, J. T., & Baeumner, A. J. (2009). PMMA bio-sensor for nucleic acids with integrated mixer and electrochemical detection. *Biosensors & Bioelectronics, 24*(8), 2428–2433. doi: 10.1016/j.bios.2008.12.025

Nunez-Forero L, L. Moyano-Ariza, H. Gait ˜ an-Duarte et al., ´ "Diagnostic accuracy of rapid tests for sexually transmitted infections in symptomatic women," Sexually Transmitted Infections, vol. 92, no. 1, pp. 24–28, 2016.

O'Neil, D., Doseeva, V., Rothmann, T., Wolff, J., & Nazarenko, I. (2011). Evaluation of Chlamydia trachomatis and Neisseria gonorrhoeae detection in urine, endo-cervical, and vaginal specimens by a multiplexed isothermal thermophilic helicase-dependent amplification (tHDA) assay. *J Clin Microbiol, 49*(12), 4121–4125. doi: 10.1128/JCM.00952-11

Parida, M., Sannarangaiah, S., Dash, P. K., Rao, P. V., & Morita, K. (2008). Loop mediated isothermal amplification (LAMP): a new generation of innovative gene amplification technique; perspectives in clinical diagnosis of infectious diseases. *Rev Med Virol, 18*(6), 407–421. doi: 10.1002/rmv.593

Pasternack, R., Vuorinen, P., & Miettinen, A. (1997). Evaluation of the Gen-Probe Chlamydia trachomatis transcription-mediated amplification assay with urine specimens from women. *J Clin Microbiol, 35*(3), 676–678.

Pasternack, R., Vuorinen, P., & Miettinen, A. (1999). Comparison of a transcription-mediated amplification assay and polymerase chain reaction for detection of Chlamydia trachomatis in first-void urine. *Eur J Clin Microbiol Infect Dis, 18*(2), 142–144.

Pickett, M. A., Everson, J. S., Pead, P. J., & Clarke, I. N. (2005). The plasmids of Chlamydia trachomatis and Chlamydophila pneumoniae (N16): accurate determination of copy number and the paradoxical effect of plasmid-curing agents. *Microbiology, 151*(Pt 3), 893–903. doi: 10.1099/mic.0.27625-0

Piepenburg, O., Williams, C. H., Stemple, D. L., & Armes, N. A. (2006). DNA detection using recombination proteins. *PLoS Biol, 4*(7), e204. doi: 10.1371/journal.pbio.0040204

Poon, L. L., Wong, B. W., Ma, E. H., Chan, K. H., Chow, L. M., Abeyewickreme, W., . . . Peiris, J. S. (2006). Sensitive and inexpensive molecular test for falciparum malaria: detecting Plasmodium falciparum DNA directly from heat-treated blood by loop-mediated isothermal amplification. *Clin Chem, 52*(2), 303–306. doi: 10.1373/clinchem.2005.057901

Quinn, T. C., Gupta, P. K., Burkman, R. T., Kappus, E. W., Barbacci, M., & Spence, M. R. (1987). Detection of Chlamydia trachomatis cervical infection: a comparison of Papanicolaou and immunofluorescent staining with cell culture. *Am J Obstet Gynecol, 157*(2), 394–399.

Redmond, S. M., Alexander-Kisslig, K., Woodhall, S. C., van den Broek, I. V., van Bergen, J., Ward, H., . . . Low, N. (2015). Genital chlamydia prevalence in Europe and non-European high income countries: systematic review and meta-analysis. *PLoS One, 10*(1), e0115753. doi: 10.1371/journal.pone.0115753

Reeve, P., Owen, J., & Oriel, J. D. (1975). Laboratory procedures for the isolation of chlamydia trachomatis from the human genital tract. *Journal of Clinical Pathology, 28*(11), 910–914.

Sabido, M., Hernandez, G., Gonzalez, V., Valles, X., Montoliu, A., Figuerola, J., . . . Casabona, J. (2009). Clinic-based evaluation of a rapid point-of-care test for detection of Chlamydia trachomatis in specimens from sex workers in Escuintla, Guatemala. *J Clin Microbiol, 47*(2), 475–476. doi: 10.1128/JCM.01975-08

Sappat, A., Jaroenram, W., Puthawibool, T., Lomas, T., Tuantranont, A., & Kiatpathomchai, W. (2011). Detection of shrimp Taura syndrome virus by loop-mediated isothermal amplification using a designed portable multi-channel turbidimeter. *J Virol Methods, 175*(2), 141–148. doi: 10.1016/j.jviromet.2011.05.013

Schachter, J. (1999). Which test is best for chlamydia? *Curr Opin Infect Dis, 12*(1), 41–45.

Schrader, C., Schielke, A., Ellerbroek, L., & Johne, R. (2012). PCR inhibitors – occurrence, properties and removal. *J Appl Microbiol, 113*(5), 1014–1026. doi: 10.1111/j.1365-2672.2012.05384.x

Schwebke, J. R., Sadler, R., Sutton, J. M., & Hook, E. W., 3rd. (1997). Positive screening tests for gonorrhea and chlamydial infection fail to lead consistently to treatment of patients attending a sexually transmitted disease clinic. *Sex Transm Dis, 24*(4), 181–184.

Scidmore, M. A., Rockey, D. D., Fischer, E. R., Heinzen, R. A., & Hackstadt, T. (1996). Vesicular interactions of the Chlamydia trachomatis inclusion are determined by

chlamydial early protein synthesis rather than route of entry. *Infect Immun, 64*(12), 5366–5372.

Shina D. J., et al (2013). *A simple integrated diagnostic platform for dna testing of chlamydia trachomatis infection*. Paper presented at the 17th International Conference on Miniaturized Systems for Chemistry and Life Sciences, Freiburg, Germany.

Singh, A. E., & Romanowski, B. (1999). Syphilis: review with emphasis on clinical, epidemiologic, and some biologic features. *Clin Microbiol Rev, 12*(2), 187–209.

Stary, A., Schuh, E., Kerschbaumer, M., Gotz, B., & Lee, H. (1998). Performance of transcription-mediated amplification and ligase chain reaction assays for detection of chlamydial infection in urogenital samples obtained by invasive and noninvasive methods. *J Clin Microbiol, 36*(9), 2666–2670.

Suttmann, H., Retz, M., Paulsen, F., Harder, J., Zwergel, U., Kamradt, J., . . . Lehmann, J. (2008). Antimicrobial peptides of the Cecropin-family show potent antitumor activity against bladder cancer cells. *BMC Urol, 8*, 5. doi: 10.1186/1471-2490-8-5

Topal, M. D., & Sinha, N. K. (1983). Products of bacteriophage T4 genes 32 and 45 improve the accuracy of DNA replication in vitro. *J Biol Chem, 258*(20), 12274-12279.Zhang, Z. F., Graham, S., Yu, S. Z., Marshall, J., Zielezny, M., Chen, Y. X., . . . et al. (1995). Trichomonas vaginalis and cervical cancer. A prospective study in China. *Ann Epidemiol, 5*(4), 325–332.

van der Helm, J. J., Sabajo, L. O., Grunberg, A. W., Morre, S. A., Speksnijder, A. G., & de Vries, H. J. (2012). Point-of-care test for detection of urogenital chlamydia in women shows low sensitivity. A performance evaluation study in two clinics in Suriname. *PLoS One, 7*(2), e32122. doi: 10.1371/journal.pone.0032122

van Dommelen, L., van Tiel, F. H., Ouburg, S., Brouwers, E. E., Terporten, P. H., Savelkoul, P. H., . . . Hoebe, C. J. (2010). Alarmingly poor performance in Chlamydia trachomatis point-of-care testing. *Sex Transm Infect, 86*(5), 355–359. doi: 10.1136/sti.2010.042598

Van Dyck, E., Ieven, M., Pattyn, S., Van Damme, L., & Laga, M. (2001). Detection of Chlamydia trachomatis and Neisseria gonorrhoeae by enzyme immunoassay, culture, and three nucleic acid amplification tests. *J Clin Microbiol, 39*(5), 1751–1756. doi: 10.1128/JCM.39.5.1751-1756.2001

van Gemen, B., van Beuningen, R., Nabbe, A., van Strijp, D., Jurriaans, S., Lens, P., & Kievits, T. (1994). A one-tube quantitative HIV-1 RNA NASBA nucleic acid amplification assay using electrochemiluminescent (ECL) labelled probes. *J Virol Methods, 49*(2), 157–167.

Vincent, M., Xu, Y., & Kong, H. (2004). Helicase-dependent isothermal DNA amplification. *EMBO Rep, 5*(8), 795–800. doi: 10.1038/sj.embor.7400200Zhang, W., Cohenford, M., Lentrichia, B., Isenberg, H. D., Simson, E., Li, H., . . . Zhang, D. Y. (2002). Detection of Chlamydia trachomatis by isothermal ramification amplification method: a feasibility study. *J Clin Microbiol, 40*(1), 128–132.

Walker, G. T. (1993). Empirical aspects of strand displacement amplification. *PCR Methods Appl, 3*(1), 1–6.

Walker, G. T., Fraiser, M. S., Schram, J. L., Little, M. C., Nadeau, J. G., & Malinowski, D. P. (1992). Strand displacement amplification – an isothermal, in vitro DNA amplification technique. *Nucleic Acids Research, 20*(7), 1691–1696.

World Health Organization, D. o. H. A. (2001). Global prevalence and incidence of selected curable sexually transmitted infections: overview and estimates.

Wozniakowski, G., & Samorek-Salamonowicz, E. (2014). Direct detection of Marek's disease virus in poultry dust by loop-mediated isothermal amplification. *Arch Virol, 159*(11), 3083–3087. doi: 10.1007/s00705-014-2157-5

Xu, C., Li, L., Jin, W., & Wan, Y. (2014). Recombinase polymerase amplification (RPA) of CaMV-35S promoter and nos terminator for rapid detection of genetically modified crops. *Int J Mol Sci, 15*(10), 18197–18205. doi: 10.3390/ijms151018197

Xu, G., Gunson, R. N., Cooper, J. M., & Reboud, J. (2015). Rapid ultrasonic isothermal amplification of DNA with multiplexed melting analysis – applications in the clinical diagnosis of sexually transmitted diseases. *Chem Commun (Camb), 51*(13), 2589–2592. doi: 10.1039/c4cc08389j

PUBLICATIONS

CURRICULUM VITAE

Name: Jekaterina Jevtuševskaja
Date of Birth: 23.10.1987
Citizenship: Estonian
E-mail: jekaterina.jevtusevskaja@ut.ee

Education

1994–2007	Tartu Puškini Secondary School, gold medal
2007–2010	University of Tartu, BSc (Gene Technology)
2010–2012	University of Tartu, MSc (Gene Technology); cum laude
2013–2017	University of Tartu, Institute of technology, doctorial studies (Engineering and Techology)

Professional employment

2013–2017	University of Tartu, SelfDiagnostics junior researcher
2012–2013	University of Tartu, specialist, laboratory of molecular biotechnology

Publications:

Kõiv, V., Andresen, L., Broberg, M., **Frolova, J.,** Somervuo, P., Auvinen, P., Pirhonen, M., Tenson, T., Mäe, A. (2013). Lack of RsmA-Mediated Control Results in Constant Hypervirulence, Cell Elongation, and Hyperflagellation in Pectobacterium wasabiae. PLoS ONE, 8 (1), e54248, journal.pone.0054248.

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. doi: 10.1016/j.jmoldx.2013.08.003.

Andresen, L., **Frolova, J.,** Põllumaa, L., Mäe, A. (2015). Dual role of RsmA in the coordinated regulation of expression of virulence genes in Pectobacterium wasabiae strain SCC3193. Microbiology-SGM, 161 (11), 2053−2264, 10.1099/mic.0.000159

Jevtuševskaja, J., Uusna, J., Andresen, L., Krõlov, K., Laanpere, M., Grellier, T., Tulp, I., and 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. doi: 10.1186/s12879-016-1674-0.

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 Jan 29:1–8. doi: 10.1080/14737159.2017.1283218.

ELULOOKIRJELDUS

Nimi: Jekaterina Jevtuševskaja
Sünniaeg: 23.10.1987
Kodkondsus: Eesti
E-post: jekaterina.jevtusevskaja@ut.ee

Haridus

1994–2007	Tartu Puškini Gümnaasium, Tartu, kuldmedal
2007–2010	Tartu Ülikool, Loodus- ja tehnoloogiateaduskond, BSc Geenitehnoloogia
2010–2012	Tartu Ülikool, Loodus- ja tehnoloogiateaduskond, MSc Geenitehnoloogia; cum laude
2013–2017	Tartu Ülikool, Loodus- ja tehnoloogiateaduskond, PhD Tehnika ja tehnoloogia, Biomeditsiini tehnoloogia, doktorant

Teenistuskäik

2013–2017	SelfDiagnostics, molekulaardiagnostika nooremteadur
2012–2013	Tartu Ülikool, spetsialist

Publikatsioonid:

Kõiv, V., Andresen, L., Broberg, M., **Frolova, J.,** Somervuo, P., Auvinen, P., Pirhonen, M., Tenson, T., Mäe, A. (2013). Lack of RsmA-Mediated Control Results in Constant Hypervirulence, Cell Elongation, and Hyperflagellation in Pectobacterium wasabiae. PLoS ONE, 8 (1), e54248, journal.pone.0054248.

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. doi: 10.1016/j.jmoldx.2013.08.003.

Andresen, L., **Frolova, J.,** Põllumaa, L., Mäe, A. (2015). Dual role of RsmA in the coordinated regulation of expression of virulence genes in Pectobacterium wasabiae strain SCC3193. Microbiology-SGM, 161 (11), 2053−2264, 10.1099/mic.0.000159

Jevtuševskaja, J., Uusna, J., Andresen, L., Krõlov, K., Laanpere, M., Grellier, T., Tulp, I., and 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. doi: 10.1186/s12879-016-1674-0.

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 Jan 29:1–8. doi: 10.1080/14737159.2017.1283218.

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