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The effect of main urine inhibitors on the activity of different DNA polymerases in loop-mediated isothermal amplification

Jekaterina Jevtuševskaja, Katrin Krõlov, Indrek Tulp and Úlo Langel

ABSTRACT

Background: The use of rapid amplification methods to detect pathogens in biological samples is mainly limited by the amount of pathogens present in the sample and the presence of inhibiting substances. Inhibitors can affect the amplification efficiency by either binding to the polymerase, interacting with the DNA, or interacting with the polymerase during primer extension. Amplification is performed using DNA polymerase enzymes and even small changes in their activity can influence the sensitivity and robustness of molecular assays.

Methods: The main purpose of this research was to examine which compounds present in urine inhibit polymerases with strand displacement activity. To quantify the inhibition, we employed quantitative loop-mediated isothermal amplification.

Results: The authors found that the presence of BSA, Mg 2+, and urea at physiologically relevant concentrations, as well as acidic or alkaline conditions did not affect the activity of any of the tested polymerases. However, addition of salt significantly affected the activity of the tested polymerases.

Conclusion: These findings may aid in the development of more sensitive, robust, cost effective isothermal amplification based molecular assays suitable for both point-of-care testing and on-site screening of pathogens directly from unprocessed urine which avoid the need for long and tedious DNA purification steps prior to amplification.

1. Introduction

A significant challenge when developing molecular assays is avoiding problems associated with the presence of interfering substances in the sample, which may significantly affect both the sensitivity of the assay and even lead to false-negative results [1]. Due to the complex nature of biological samples, most samples typically contain several components that inhibit a given amplification reaction. These compounds may interfere during cell lysis, through direct interaction with either DNA or DNA polymerases, or by reducing the quality or quantity of template DNA [2]. Inhibitors can also originate from insufficiently purified target DNA or even from reaction components. A large number of commercially available rapid DNA extraction methods exist, however, only a few of these enable one to efficiently recover inhibitor-free DNA, and typically depend on the isolated material [3,4]. Because of this, whenever genomic DNA is extracted from complex biological samples, one should verify that no traces of inhibitory compounds remain in the extract prior to performing amplification. Despite intensive efforts to eliminate inhibitors from samples during purification, it is still advantageous to search for inhibitor resistant polymerases to avoid time consuming and often tedious DNA purification methods. Finding rapid polymerases with improved inhibitor tolerance that are capable of direct amplification of DNA from clinical samples would both simplify and reduce the analysis time, and significantly reduce the final cost of the diagnostic tool.

Currently, the main detection techniques used for target DNA amplification are PCR and isothermal amplifications. Despite the fact that PCR is the most common DNA amplification method, it is also very sensitive to various inhibitors in crude samples, and is therefore mainly suitable for centralized hospital facilities that are able to implement complicated workflows using more expensive laboratory apparatus which are required to extract genetic material from the crude sample prior to amplification. Various isothermal amplification methods such as strand displacement amplification [5], rolling circle amplification [6], helicase-dependent amplification [7], cross priming amplification [8], and loop-mediated isothermal amplification (LAMP) [9] have recently been developed and successfully applied in medical diagnostics. Compared with PCR, they do not require a thermal cycler because they are carried out at constant temperature due to the strand displacement activity of DNA polymerases and can show a good tolerance to the inhibitors [10–13]. Taken together, this make isothermal amplification methods very appealing for the diagnosis of infectious disease because they provide the possibility of developing simple nucleic acid tests for field use or point-of-care diagnostics in low to
middle income countries where the provision of DNA extraction may be either prohibitively expensive or impractical.

The goal of this study was to compare commercially available polymerases that possess strand displacement activity to determine which are the most rapid and urine tolerant when utilized using current diagnostic tools. Several reports describe the successful application of LAMP in the development of rapid and sensitive diagnostic assays for pathogen detection and nucleic acid analysis [14]. LAMP has been shown to detect pathogens from samples that have been lightly processed, or clinical samples such as urine [10], faeces [15], sputum [11], or blood [12]. LAMP is a relatively new isothermal amplification technique that is able to detect smaller amounts of DNA than PCR-based tests [10,16,17] because of its sensitivity, specificity, and high tolerance to various inhibitors. The most commonly used replicative enzyme in LAMP assays is Bst polymerase, derived from Bacillus stearothermophilus. This enzyme contains 5’→ 3’ polymerase activity, but lacks 5’→3’ exonuclease activity [18]. Many ways of employing Bst and other commercially available polymerases have been explored in order to increase amplification speed, yield, or inhibitor resistance. During this study we tested the efficiency of the following DNA polymerases 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 known inhibitors typically found in urine samples at physiologically relevant concentrations. For that, we used a recently optimized LAMP-based detection assay [10]. Our intention was to provide a solid basis upon which one may develop more sensitive, robust, and cost-effective molecular diagnostic platforms able to accurately detect pathogens directly from unprocessed samples using a method suitable for large-scale application.

2. Patients and methods

2.1. DNA standard

A standard curve was constructed using serial dilutions of BglII linearized pGL3-CDS2 plasmid [19] at concentrations of 10^{8}, 10^{7}, 10^{6}, 10^{5}, and 10^{4} template copies per reaction with four parallel reactions.

2.2. Quantitative LAMP reaction

Each LAMP reaction was performed as previously described [10] with 0.5 µl EvaGreen (Biotium) fluorescent dye and 0.3 µl ROX (Thermo Fisher Scientific Inc., Carsbad, CA, USA) reference dye added for quantitative analysis. Amplification primers were not carrying Biotin/FAM labeling and reaction products were analyzed using an Applied Biosystems 7900HT Real-Time PCR System. All qLAMP reactions were performed at 63°C with 40 cycles, each 1 min long, data reading after 30 s.

Data analysis was evaluated by Sequence Detection Software (SDS) version 2.4 (Life Technologies Ltd, Paisley, UK) with manual baseline/threshold settings to assess the threshold time (Tt). Urine and inhibitor tolerance results were expressed as estimated time to result by normalizing them to the results of Bsm polymerase [10]. Water control assays were included where the template was omitted to evaluate the presence of contamination; all water control assays produced no detectable Tt signal. Each data point is representative of at least two independent experiments.

2.3. LAMP reaction for lateral flow detection

Each LAMP reaction was performed as previously described [10] with reaction total volume 50 µl. All LAMP reactions for lateral flow detection were performed at 63°C for 1 h. Amplification primers were carrying Biotin (FIP and LF primers) and FAM (BIP and LB primers) labeling, enabling biotin-FAM double-labeled Chlamydia trachomatis specific product detection on the lateral flow strips (AMODIA Bioservice GmbH). Result visualization was performed using lateral flow detection strips that enable an answer to be obtained within 10 min using 10^{5} pGL3-CDS plasmid template copies per reaction. For that reaction, products were diluted two times in distilled water and analyzed on lateral flow strips, where T indicates the test band or presence of biotin-FAM-labeled reaction product and C indicates the lateral flow control band.

2.4. LAMP DNA polymerases

16U Bsm polymerase (Thermo Fisher Scientific); 16U Bst/Bst2.0/Bst2.0WarmStart polymerases (NEB); 1× OmniAmp (Lucigen); 16U GspSSD/GspSSD2.0/GspM/GspM2.0/Tin (Optigen); 40U SD polymerase (Bioron) were used in qLAMP per reaction (Table 1).

2.5. Urine inhibitor preparation

To study the effect of various urine inhibitors on the amplification capacity of the 11 polymerases, different concentrations of NaCl (150 and 300 mM); MgSO_{4} (1, 5, and 10 mM); BSA (0.7, 2, 7, 20, and 40 mg/ml); urea (15, 150, 300 mg/l); HCl; 0.00001 M NaOH or pooled urine (5%, 10%, 15%, 20%) were added to qLAMP reactions. Five microliters of each inhibitor was mixed with template DNA at concentrations ranging between 10^{5} and 10^{8} and then directly added into the qLAMP reaction.

2.6. Pooled human urine

Pooled urine was used to conduct a sensitivity analysis. C. trachomatis negative normal human urine was collected fresh from five men and five women and pooled immediately in equal volumes.

2.7. qLAMP reaction conditions

The reaction conditions for all 11 polymerase were changed and optimized accordingly to the advice from the supplier and our test analysis. In total, we analyzed 11 polymerases with strand displacement activity and compared the results with a previously optimized qLAMP-based assay [10]. For that reason, all polymerase reactions were carried out at 63°C to compare their time to result to qLAMP assay with Bsm polymerase. In Table 1, we present a list of polymerases tested in this study and the reaction conditions experimentally estimated for these polymerases (for SD and OmniAmp polymerases reaction conditions were optimized experimentally).
3. Results

3.1. Analysis of LAMP polymerases activity

In order to evaluate the efficiency of different LAMP polymerases under optimized conditions (Table 1) we tested them in water in the presence of different Chlamydia trachomatis CDS2 copy numbers from $10^8$ to $10^4$ at 63°C. We have previously determined that the optimum amplification time for Bsm polymerase is 21 min [10]. In this study, we analyzed the optimum amplification time of the other polymerases and compared them with Bsm.

Our results show that GspSSD2.0 polymerase is significantly faster (two times) than Bsm polymerase, however, it always produced an unspecific background after 30 min of amplification even in the absence of template DNA and using the supplier recommended concentration of MgSO$_4$ (5 mM) and dNTP (0.4 mM). The polymerases GspM2.0, GspSSD, Bst2.0, Bst2.0, and WarmStart also displayed good amplification because their reaction speed were slightly better compared with Bsm polymerase. OmniAmp and Bst were as good as Bsm polymerase. GspM polymerase was slightly slower than Bsm polymerase (ranging between 21 and 25.2 min). Both SD and Tin polymerases were significantly slower than Bsm polymerase (Supplemental Figure 1). Based on this data, polymerases such as GspM2.0; GspSSD2.0; GspSSD, Bst2.0, Bst2.0, WarmStart, Bsm, OmniAmp, and Bst are the fastest polymerases and all are able to generate detectable amplification product within 25 min.

### Table 1. List of polymerases and reaction conditions applied in this study.

<table>
<thead>
<tr>
<th>Polymerase</th>
<th>Tested reaction conditions</th>
<th>Polymerase name</th>
<th>Tested reaction conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bsm</td>
<td>20 mM Tris–HCl pH 8.8 10 mM KCl, 10 mM (NH$_4$)$_2$SO$_4$ 8 mM MgSO$_4$ 0.1% Tween-20 1.4 mM dNTP 0.8 M betain 16 U/rxn polymerase</td>
<td>GspSSD</td>
<td>50 mM Tris–HCl pH 8.1 30 mM KCl, 30 mM (NH$_4$)$_2$SO$_4$ 0.1% Triton X-100 1 M betain 1.4 mM dNTP 8 mM MgSO$_4$</td>
</tr>
<tr>
<td>Bst</td>
<td>20 mM Tris–HCl pH 8.8 10 mM KCl, 10 mM (NH$_4$)$_2$SO$_4$ 0.1% Triton X-100 1.4 mM dNTP 8 mM MgSO$_4$ 0.8 M betain 16 U/rxn polymerase</td>
<td>GspSSD2.0</td>
<td>50 mM Tris–HCl pH 8.1 30 mM KCl, 30 mM (NH$_4$)$_2$SO$_4$ 0.1% Triton X-100 1 M betain 1.4 mM dNTP 8 mM MgSO$_4$</td>
</tr>
<tr>
<td>Bst2.0</td>
<td>20 mM Tris–HCl pH 8.8 10 mM (NH$_4$)$_2$SO$_4$ 50 mM KCl, 8 mM MgSO$_4$ 0.1% Tween-20 1.4 mM dNTP 0.8 M betain 16 U/rxn polymerase</td>
<td>GspM</td>
<td>20 mM Tris–HCl pH 8.8 10 mM KCl, 10 mM (NH$_4$)$_2$SO$_4$ 0.01% Triton X-100 1 M betain 1.4 mM dNTP 8 mM MgSO$_4$</td>
</tr>
<tr>
<td>Bst 2.0 WarmStart</td>
<td>20 mM Tris–HCl pH 8.8 10 mM (NH$_4$)$_2$SO$_4$ 50 mM KCl, 8 mM MgSO$_4$ 0.1% Tween-20 1.4 mM dNTP 0.8 M betain 16 U/rxn polymerase</td>
<td>GspM2.0</td>
<td>20 mM Tris–HCl pH 8.8 10 mM KCl, 10 mM (NH$_4$)$_2$SO$_4$ 0.01% Triton X-100 1 M betain 1.4 mM dNTP 8 mM MgSO$_4$</td>
</tr>
<tr>
<td>Tin exo-LF</td>
<td>30 mM Tris–HCl pH 8.8 30 mM KCl, 10 mM (NH$_4$)$_2$SO$_4$ 0.1% Triton X-100 1 M betain 1.4 mM dNTP 8 mM MgSO$_4$ 16 U/rxn polymerase</td>
<td>SD</td>
<td>1× buffer (Tween 20 and BSA) 8 mM MgCl$_2$ 0.8 M betain 0.5 mM dNTP 40U polymerase</td>
</tr>
<tr>
<td>OmniAmp</td>
<td>20 mM Tris–HCl pH 8.0 10 mM (NH$_4$)$_2$SO$_4$ 10 mM KCl, 0.1% Triton X-100 1.4 mM dNTP 0.8 M betain 1× polymerase</td>
<td></td>
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</tr>
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</table>

3.2. Effect of the urine on the amplification speed of LAMP polymerases

Because DNA purification/concentration steps are too complicated to use in POC assays it would be advantageous if the amplification method would tolerate as high a percentage of urine in the reaction mixture as possible. This would allow one
to increase the sensitivity of the assay and simplify the procedure of detecting pathogens. For that reason, we tested the urine-tolerance of 11 different LAMP polymerases. In our experimental set-up, each qLAMP reaction mixture contained between 0% and 20% of urine (final concentration). While many LAMP-based assays purify genomic DNA from the sample to ensure better sensitivity, only a few reports have shown that LAMP can detect pathogens directly from poorly or non-processed crude samples [10,16,20]. Our results demonstrate that Bsm polymerase is able to tolerate up to 20% urine in the final reaction volume while having no influence on the amplification time (Figure 1). DNA polymerases such as GspM, GspM2.0, Bst, and Bst2.0 are more sensitive to the presence of urine in the reaction and are able to tolerate up to 10% urine without significantly affecting the amplification time. For Bst2.0 WarmStart and GspSSD2.0 polymerases addition of 5% of urine had no effect on reaction efficiency, however, the addition of 10% of urine prolonged the reaction by up to 10 min. The worst polymerases in terms of urine tolerance were SD, OmniAmp, Tin, and GspSSD and therefore we conclude that they are less applicable for the direct detection of pathogens in urine. GspSSD polymerase displayed a prolonged amplification time of up to 19 min in the presence of 10% urine. Tin polymerase in the presence of 5% of urine displayed an amplification speed of up to 21 min. SD polymerase was also able to tolerate up to 5% of urine without significant prolongation of amplification time. Omni Amp was very sensitive to the presence of urine and was not able to tolerate even 5% urine in the reaction (Figure 1). These qLAMP results correlate well with lateral flow LAMP results (Supplemental Figure 6) and show that LF-LAMP can offer on-site cost-efficient screening possibility and therefore can be easily applied as a POC test. However we saw again that GspSSD2.0 polymerase produced unspecific background signal after 1 h of amplification in the absence of template DNA which also correlate well with qLAMP results, making GspSSD2.0 polymerase unfavorable for lateral flow detection at tested conditions.

3.3. Effect of the urea on the LAMP DNA polymerases

Due to the fact that the amplification sensitivity can be dramatically reduced/inhibited by the presence of crude urine in the reaction (Figure 1), it would be advantageous to identify the main inhibitors in urine. Previously, Hedman and Rådström have shown that urea prevents the noncovalent bonding of polymerase enzymes and interferes with primer annealing, thereby inhibiting amplification [21]. Based on this knowledge, urea has become a primary candidate for affecting the amplification sensitivity [22]. Therefore, we went on to investigate the impact of urea on the activity of DNA polymerases. It has been reported that the average concentration of urea in first-void urine can be up to 300 mM (or 18 mg/ml) [22]. This means that if 10–20% urine is added to the LAMP reaction, the final urea concentration in reaction would be 30–60 mM. Our results show that concentrations even greater than 66.6 mM do not affect the amplification time and the activity of all polymerases we tested remained at the same level (Supplemental Figure 2). Our results support the previously published results which demonstrated that polymerases with strand displacement activity can tolerate relatively high concentration of urea (even up to 1.8 M), which indicates an increased robustness of the LAMP assay to urea mediated inhibition [23]. Conversely, PCR is more susceptible to the presence of urea and displays inhibition already at concentrations ≥50 mM [22,24]. We observed that the suppressive effect of urine has no correlation with the concentration of urea. Based on that observation, we can assume that urea is not the main inhibitor in LAMP reactions.

3.4. Effect of BSA on LAMP DNA polymerases

The concentration of albumin in human urine depends on the patient’s medical history and is normally less than 10 mg/l. Microalbuminuria or macroalbuminuria typically occurs at concentrations between 20 and 200 mg/l and occasionally is more than 200 mg/l in urine. The average total protein concentration in urine has been observed to be 1–14 mg/dl or less than 100 mg/day. Based on that knowledge, we went on to test how different BSA concentrations can affect the ability of DNA polymerases to amplify. It has already been shown that the addition of BSA alone or when used in combination with organic solvents can significantly enhance amplification yield [25]. Our results show that all polymerases we tested are resistant to the average concentration of BSA in urine and their activity remained at the same level even when higher amounts of BSA were added (Supplemental Figure 3). While BSA does not adversely affect the ability to amplify DNA, it

![Figure 1. Inhibitory effect of different urine concentrations (0–20%) on the amplification time of 11 strand displacement DNA polymerases (min). These results were analyzed quantitatively, and the remaining amplification time was calculated as the average time compared to the reaction in the absence of urine at standard dilutions of between 10^8–10^4 target copies per reaction. * no amplification was detected.](image-url)
could potentially be used to improve assay sensitivity due to its binding capacity for lipids and other organic molecules that may act as inhibitors.

3.5. Effect of pH on LAMP DNA polymerases

Many enzymes are sensitive to pH and thus have optimal activity over a narrow range of pH values. Changes in pH can alter intermolecular bonds, which not only changes the conformation and effectiveness of the enzyme itself, but also the speciation and effective concentration of co-factors. The optimal pH for a given enzyme depends on the environment it is present in. It has been already shown that the nucleases normally present in urine can significantly reduce assay sensitivity because they are able to degrade template DNA at pH range 6–7 [26]. It is thus essential to immediately perform DNA analysis on urine samples without long-term storage in order to prevent target DNA from nuclease degradation.

Normally, urine is slightly acidic or alkaline with a pH ranging from between 4 and 9. Although all polymerases are supplied with a buffer (pH 8–9) to minimize changes in pH, we tested how the addition of acidic or alkaline component affects the activity of each polymerase. Our results show that the activities of the polymerases remain at the same level when acidic or alkaline solution was added to the qLAMP reaction (Supplemental Figure 4).

3.6. Effect of magnesium on LAMP DNA polymerases

The concentration of magnesium has a significant impact on the efficiency and specificity of DNA amplification: it directly influences both polymerase activity and fidelity, and affects the annealing temperature of the various hybrids that can form during amplification, for instance primer-template, template-template, and primer-primer. In the current study we examined how the addition of magnesium to urine at physiologically relevant concentrations can affect the activity of each polymerase. Because the average magnesium concentration in urine ranges between 1.7 and 5.7 mmol/l, we added 0.1, 0.5, and 1 mM of magnesium to the urine in our studies. Our results show that 0.5 mM of magnesium ions does not affect any of the polymerases we tested. However, at higher concentrations of MgSO4, near 1 mM, we observed a slight increase in the amplification time (up to 4 min) for Bst2.0 Warm Start, Tin, and Bst2.0 polymerases (Supplemental Figure 5).

3.7. Effect of salt on LAMP DNA polymerases

Because the average concentration of Na+ ions in the first-void urine ranges between 54 and 190 mM, we tested how the addition of NaCl can affect the activity of each polymerase. For this, we added NaCl to the urine samples until the average total amount of salt was either 150 or 300 mM and proceeded to test the activity of each polymerase. Both Bsm and GspM2.0 were able to tolerate between up to 45 mM NaCl without influencing their sensitivity. Interestingly, the addition of salt even slightly accelerated the speed of the Bsm polymerase. GspSSD2.0 polymerase was also less affected by a high salt concentration. Polymerases such as GspM, Bst, Bst2.0, Bst2.0 WarmStart, OmniAmp, and GspSSD were able to tolerate up to 15 mM of NaCl. However, GspM, Bst, Bst2.0, and Bst2.0 WarmStart polymerases displayed a prolonged amplification time between 12 and 26 min upon addition of 45 mM NaCl. GspSSD polymerase also performed poorly as its amplification time was prolonged up to 26 min in the presence of 45 mM NaCl (Figure 2). The worst polymerase in terms of salt tolerance was SD polymerase (even 15 mM of NaCl prolonged amplification time by up to 10 min). Both Tin and OmniAmp polymerases in the presence of 30 mM NaCl displayed a prolonged amplification of up to 23 min. Effect of salt on LAMP DNA polymerases were also analyzed with lateral flow LAMP detection method and the results compared to those obtained using qLAMP detection method (Supplemental Figure 7). This data shows that salt is one of the most critical components in urine that can dramatically reduce the sensitivity of molecular assays (Figure 1).

4. Discussion

The detection and identification of pathogens within complex biological samples using DNA amplification can be

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**Figure 2.** Influence of physiologically relevant NaCl concentrations on the amplification time (min) of 11 different strand displacement DNA polymerases in urine. These results were analyzed quantitatively, and the remaining amplification time was calculated as an average time compared to the reaction in the absence of salt at standard dilutions of between 10^8 to 10^4 target copies per reaction. Note that only three of the enzymes tested were not adversely affected by the presence of NaCl.
challenging due to presence of common inhibitors that directly or indirectly affect the polymerase activity. The inhibitors present in crude samples are a heterogeneous class of substances that can dramatically reduce the sensitivity of diagnostic assays and may even lead to false-negative results. This, in turn, leads to inaccurate target quantification and an underestimation of the true number of targets present in the sample [27]. The most common solution to overcome this problem is to purify the genomic DNA in the biological sample using tedious and time-consuming extraction and purification methods. However, this strategy does not guarantee an inhibitor-free sample and is often accompanied with a significant loss in template DNA even when the most efficient method is employed. In addition, purification methods are less applicable for POC assays which aim to reduce the prevalence of undiagnosed infections by providing rapid and efficient diagnosis. Another option can be utilization of a polymerase that is less susceptible to various inhibitors. This last option can be easily applied at POC and thereby would improve the accuracy of DNA assays, and result in providing a rapid, cost-effective, sensitive, and specific method to detect pathogens.

LAMP has been previously shown to exhibit high analytical sensitivity and specificity in the detection of various infectious pathogens [12,15–17,24]. This method has many advantages: amplification takes place at isothermal conditions; DNA/RNA amplification can take place directly in crude samples; amenable to simple product detection (turbidity, fluorescence dyes, electrochemical detection). Taken together, all of these advantages point toward LAMP being a useful method in resource limited settings and compatible with the ASSURED format [28]. LAMP was developed in the year 2000 year by Notomi and his colleagues and originally used Bst DNA polymerase with strand displacement activity for isothermal amplification [18]. Since this time, various polymerases with strand displacement activity and their derivatives with different properties have been developed and are widely used for more efficient isothermal amplification [29–31]. The direct detection of pathogens in crude unpurified samples is challenging due to the complex nature of biological samples, however, a few reports have shown that the LAMP reaction can be performed on crude samples. For example, Kaneko et al. demonstrated that Bst polymerase can tolerate 30% of saline, PBS, or MEM; 20% of vitreous and 1% of urine, serum, or plasma [32]. In 2014, Chander described the application of a novel OmniAmp polymerase for rapid RT-LAMP and DNA LAMP amplification that was less affected by the use of untreated blood samples [30]. In addition, Woźniakowski et al. demonstrated that GspssD polymerase is able to amplify target DNA directly from poultry dust samples [33]. Tian et al. demonstrated that Bst3.0 polymerase is able to detect the Zika virus in 20% serum [34]. However, most reports still use loop-mediated amplification for the detection of DNA already extracted and purified from biological samples.

In this study, we evaluated the urine-tolerance of 11 polymerases that possess strand displacement activity in order to determine which are the most tolerant to inhibitors and applicable for the direct detection of pathogens from crude samples. First, we evaluated the relative speed of these polymerases in water (was expressed as relative time to result as compared to Bsm). Good results were obtained with GspM2.0, GspSSD2.0, GspSSD, Bst2.0, Bst2.0 WarmStart, Bsm, OmniAmp, and Bst polymerases as they were able to amplify target DNA already within 25 min in water. SD and Tin polymerases were significantly slower than the other polymerases and amplification took longer than 33 min. Our results demonstrate that urine can significantly affect polymerases activity by reducing the sensitivity and performance time of the assay. We further demonstrated that the most urine tolerant polymerase is Bsm, which is able to tolerate 20% urine without significant change of the reaction speed. DNA polymerases such as GspM, GspM2.0, Bst, and Bst2.0, are more susceptible to the presence of urine in the LAMP reaction and are able to tolerate up to 10% urine without significantly affecting the performance time. Bst2.0WarmStart and GspSSD2.0 polymerases in the presence of 10% of urine displayed a prolonged amplification time of up to 10 min. We found that the least urine tolerant polymerases are OmniAmp, Tin, SD, and GspSSD and we conclude that these are less applicable for the direct pathogen detection in urine. Interestingly, we showed that the main PCR inhibitor, urea, has no inhibitory effect on tested polymerases and all polymerases were able to tolerate a relatively high amount of urea (up to 66.6 mM) in the sample, thereby demonstrating that LAMP assay is not inhibited by physiological levels of urea. Physiological levels of both BSA and magnesium also do not impact on the amplification time of all polymerases tested. We also showed that the activities of all 11 polymerases remained unchanged when either an acidic or alkaline solution was applied to the LAMP reaction. However, at the same time changes in pH values can have a significant impact on the efficiency and specificity of the amplification: nucleases that are normally present in urine can significantly reduce assay sensitivity because they are able to degrade template DNA over the pH range 6–7, which makes long-term storage of urine unfavorable.

We found a good correlation between the urine tolerance and salt tolerance of polymerases. All polymerases, which were sensitive to the presence of urine also display a reduction in amplification time when NaCl concentration was increased in the reaction. Therefore, we suggest that the best polymerase in terms of urine and salt tolerance for LAMP assays is Bsm polymerase. Although it is not as fast as GspSSD2.0, Bsm polymerase is able to tolerate up to 20% urine and its speed was even slightly improved in the presence of high concentrations of NaCl (15–45 mM). GspM2.0 tolerates inhibitors in a similar way as Bsm polymerase where addition even 45 mM NaCl did not affect the amplification time and the addition of up to 10% urine did not affect the speed of the polymerase reaction. GspM2.0 polymerase is also slightly faster than Bsm polymerase when the reaction is carried out in water (17.2 vs. 21 min). GspM, Bst, Bst2.0, and Bst2.0 WarmStart polymerases can be used directly in LAMP assays for direct amplification of genomic DNA from urine sample because they all are able to tolerate high amount of Na ions without significant prolongation of amplification time in the presence of 30 mM NaCl (4 min delay for Bst; 6 min for GspM/ Bst2.0 and 8 min for Bst2.0 WarmStart). The addition of 10% of urine does not significantly affect the amplification time of GspM, Bst, and Bst2.0 polymerases; however, for GspSSD2.0 and Bst2.0...
WarmStart polymerases, amplification is prolonged for 7–10 min. The worst polymerases in terms of salt and urine tolerance are Tin, OmniAmp, GspSSD, and SD polymerases (delay of up to 15–23 min in the presence of 30 mM NaCl for Tin, OmniAmp, GspSSD polymerases and presence of 5% of urine significantly increased the amplification time (OmniAmp were not even able to tolerate 5% of urine in the reaction).

5. Conclusion
In summary, we have demonstrated that polymerases have different tolerance toward urine and urine components in the reaction, and the most significant factor is the concentration of salt found in crude urine samples. Thus, polymerases such as Bsm and GspM2.0 are the most urine tolerant polymerases for isothermal amplification and provide rapid and efficient amplification of target DNA directly without affecting amplification time. However, polymerases such as GspM, Bst, Bst2.0, Bst2.0 WarmStart, and GspSSD2.0 can also be employed to amplify genomic DNA directly from urine because they can tolerate between 30 and 45 mM of NaCl and between 10% and 15% urine in the reaction volume. One possibility to increase the tolerance of polymerase reactions in untreated biological samples is to eliminate high Na ion concentrations from the reaction. Our findings allow us to conclude that one-step amplification using untreated urine using a urine tolerant polymerase may eliminate the need to purify DNA and could improve the clinical utility of these methods in POC settings while providing a sensitive, fast, and efficient method of detecting pathogens directly from crude urine samples. This work could form the basis for the development of more rapid and sensitive molecular diagnostic tests that can be conducted near or at the location of the patient care thereby reducing the delay between diagnosis and the initialization of treatment.

Key issues
• Polymerases such as GspM2.0; GspSSD2.0; GspSSD, Bst2.0, Bst2.0, WarmStart, Bsm, OmniAmp, and Bst are the fastest polymerases and all are able to generate detectable amplification product within 25 min.
• Presence of BSA, Mg$^{2+}$, and urea at physiologically relevant concentrations, as well as acidic or alkaline conditions did not affect the activity of any of the tested polymerases with a strand displacement activity.
• Bsm polymerase is able to tolerate up to 20% urine in the final reaction volume; GspM, GspM2.0, Bst, and Bst2.0 are more sensitive to the presence of urine in the reaction and are able to tolerate up to 10% urine without significantly affecting the amplification time; for Bst2.0 WarmStart and GspSSD2.0 polymerases addition of 5% of urine had no effect on reaction efficiency; SD, OmniAmp, Tin, and GspSSD were the worst polymerases in terms of urine tolerance.
• We found that the polymerases Bsm and GspM2.0 had the highest level of salt tolerance and are able to amplify target DNA in the presence of up to 45 mM NaCl without any reduction in amplification activity.
• Conversely, we found that the polymerases 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.
• Polymerases such as GspSSD, GspSSD2.0, Bst2.0, Bst2.0 WarmStart and GspM are able to tolerate up to 15 mM NaCl without significant prolongation of amplification time.

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Declaration of interest
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