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Nucleic acid extraction from complex biofluid using toothpick-actuated over-the-counter medical-grade cotton

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ABSTRACT

Nucleic acid amplification technique (NAAT)-assisted detection is the primary intervention for pathogen molecular diagnostics. However, NAATs such as quantitative real-time polymerase chain reaction (qPCR) require prior purification or extraction of target nucleic acid from the sample of interest since the latter often contains polymerase inhibitors. Similarly, genetic disease screening is also reliant on the successful extraction of pure patient genomic DNA from the clinical sample. However, such extraction techniques traditionally utilize spin-column techniques that in turn require centralized high-speed centrifuges. This hinders any potential deployment of qPCR- or PCR-like NAAT methods in resource-constrained settings. The development of instrument-free nucleic acid extraction methods, especially those utilizing readily available materials would be of great interest and benefit to NAAT-mediated molecular diagnosis workflows in resource-constrained settings. In this report, we screened medical-grade cotton, a readily available over-the-counter biomaterial to extract genomic DNA (gDNA) spiked in 30 %, 45 %, and 60 % serum or cell lysate. The extraction was carried out in a completely instrument-free manner using cotton and a sterilized toothpick and was completed in 30 min (with using chaotropic salt) or 10 min (without using chaotropic salt). The quality of the extracted DNA was then probed using PCR followed by agarose gel analysis for preliminary validation of the study. The qPCR experiments then quantitatively established the extraction efficiency (0.3–27 %, depending on serum composition). Besides, percent similarity score obtained from the Sanger sequencing experiments probed the feasibility of extracted DNA towards polymerase amplification with fluorescent nucleotide incorporation. Overall, our method demonstrated that DNA extraction could be performed utilizing toothpick-mounted cotton both with or without using a chaotropic salt, albeit with a difference in the quality of the extracted DNA.

1. Introduction

Nucleic acid amplification tests (NAATs) such as qPCR have remained the primary intervention for the detection and containment of a pathogen-mediated disease outbreak and also for screening genetic diseases, food soiling, and biowarfare prevention. The utility of NAATs is, however, dependent on the quality of nucleic acid templates being used, where the presence of DNA polymerase inhibitors may affect its efficacy as well as accuracy.[1] Silica-based spin-column kits, magnetic particles, or automated extraction-amplification systems could be utilized to extract nucleic acid from the sample of interest. Despite established sensitivity, automated extraction systems remain expensive with high per sample cost[2], while silica-based spin-column kits require centralized high-speed centrifuges. Commercial magnetic particles, either silica or carboxyl coated, or pH-modulated charge-switching chitosan-based, are costly. Their in-house preparation, although low cost as shown in bio-on-the-magnetic beads (BOMB) initiative or through minimal instrument intensive synthesis[3,4], still require

Abbreviations: NAAT, nucleic acid amplification test; NTC, no template control; qPCR, quantitative real time PCR; C_t value, cycle threshold value; FBS, fetal bovine serum.

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precursor chemicals (such as methacrylic acid[3], triethyl orthosilicate,[3] chitosan,[4–5] Fe (II) and Fe (III) salts[3,4,6] and alkaline solution,[3] instruments (magnetic stirrer-hot plate,[3–4] and at least 7–24 h of preparation time,[3–4].[6] Although felicitous for fast and mostly instrument-free nucleic acid extraction, such magnetic particle preparation is difficult to execute in limited-resource settings.

The requirement of instrument-free methods suitable for limited-resource settings has led to the development and innovation of alternative techniques of nucleic acid extraction. This includes the use of filter paper that enable nucleic acid extraction from various matrices as well as a filter paper fitted device for the same,[7] in-house preparable spin-column using filter papers[8], and pipette-actuated magnetic beads among others[9]. Table S1 provides a comprehensive list of methods that are suitable for nucleic acid extraction without utilizing heavy and centralized equipments such as high-speed centrifuges. It also includes nature of nucleic acid extracted, complex biofluid present in the sample of origin, cost associated (when available), assay time, and downstream NAAT. The entries in the table can be broadly categorized into three segments. Entries 1–6 predominantly are free of equipments at the actual extraction steps[10–15]. Entries 7–10 utilize magnetic separation in various capacities[4,9,16,17]. The rest of the entries (11–28) utilizes microfluidics[8,18–34]. However, entries 1–5 require sophisticated instruments for the fabrication of the extraction system with the exception of entry 6, that utilizes unmodified but non-readily available glass fiber paper itself. Similarly, magnetic extraction systems in entries 7–10 require magnetic beads necessitating prior preparation or procurement. The extraction systems comprising of microfluidic devices, although simple to handle, require either syringe pump or centrifugation for function. While these platforms have greatly advanced the innovations necessary for a limited-resource NAAT detection, neither thus use readily available materials or equipment for preparing a nucleic acid extraction system. This would especially be of significant relevance in a developing country where items such as filter paper for fabricating a spin column are relatively unavailable.

Like silica, cellulose displays a negative surface charge over a large pH range[35]. Similarly, cellulose-based Whatman filter paper discs have been deployed in the extraction of genomic DNA from plant extracts and complex biofluid[7]. It was therefore anticipated that cotton, primarily composed of cellulose would be adept in extracting DNA from complex biofluid solution in presence of chaotropic salt. In this study, we present our investigation on a nucleic acid extraction platform using readily available over-the-counter medical-grade cotton with disposable toothpicks as an actuator. Their application in extracting *Escherichia coli* (*E. coli*) or *Staphylococcus aureus* (*S. aureus*) genomic DNA nucleic acid spiked in 30 %, 45 %, and 60 % serum (v/v) or cell lysate has been investigated. The role of chaotropic salts in extracted nucleic acid quality and extraction efficiency has been probed. The quality and efficiency of the extracted nucleic acid have been analyzed using PCR-gel electrophoresis, qPCR, and sequencing. Additionally, the possible role of PCR inhibitors in post-extraction NAAT efficiency was analyzed through A280 measurements.

2. Results and discussion

2.1. Study methodology

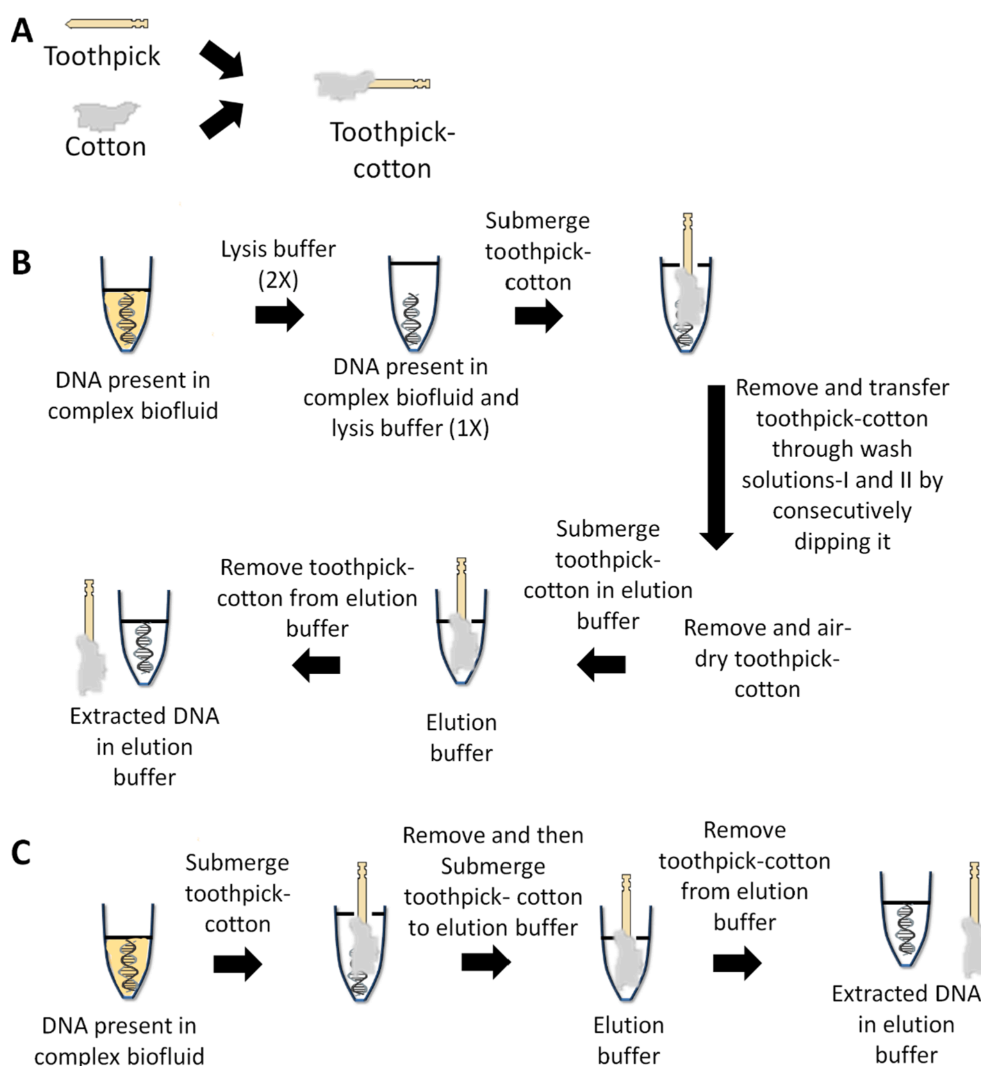
The objective of this study is to find and investigate readily available materials as well as methods that could enable nucleic acid extraction from complex biofluids involving minimal or no instruments. The material used in this study, medical-grade over-the-counter cotton, was taken up for its cellulose composition enabling surface negative charge[35] and ready availability at any medical shop as well as homes as a first-aid item. As we intended to develop an instrument-free or minimal instrument-intensive extraction method, the cotton either needed to be fitted to a flow device that would accommodate the nucleic acid binding, washing, and elution steps. Alternatively, an external actuator might transfer it from one solution to the other that would enable these steps. In this study, we adopted the latter model using a wooden toothpick, another very commonly available household item (Scheme 1A, Fig. 1). The toothpicks were sterilized through autoclaving, made single use only, and thus disposed of after every extraction workflow. If required, more stringent sterilization enabling DNA/RNA or DNase/RNase removal could be achieved through treatment with 0.4–10 % bleach (sodium hypochlorite) with or without involving UV and 70 % ethanol treatment[36].

Like silica-based kits, negatively charged cotton surfaces are therefore expected to be benefitted from chaotropic salt-assisted nucleic acid binding, leading to the incorporation of guanidium chloride in this study (Scheme 1B). As in conventional silica-based extraction methods, this would involve exposure of the nucleic acid-containing solution to a chaotropic salt, binding to cotton, washing away non-nucleic acid molecules, alcohol-mediated de-salting (washing), and elution to a low salt buffer. However, chaotropic salts such as guanidium hydrochlorides (Gu-HCl), although inexpensive, are not commonly available. Therefore, we also investigated if cotton could directly bind to nucleic acid present in a complex biofluid (without a chaotropic salt), bypass the alcohol and chaotropic salt-based washing steps, and directly release it to an aqueous elution buffer (Scheme 1C). The latter method has been termed direct extraction in this manuscript. 7.1 ng/μL (ca. 1.4×10^6 copies/μL) of the *Escherichia coli* (*E. coli*) genomic DNA (gDNA) in 140 μL aqueous solution or 30, 45, and 60 % fetal bovine serum spiked solution was employed as the extraction target for the proposed workflows. To explore the compatibility of the extraction technique with cell lysate, purification of *S. aureus* genomic DNA from *E. coli* lysate using the same workflows was investigated.

2.2. Preliminary investigation into DNA extraction from 30 to 60 % serum and analysis of extracted DNA through PCR-gel electrophoresis

The feasibility of the proposed extraction method was first investigated using cotton-based extraction of *E. coli* gDNA from an aqueous solution. Post-extraction, the gDNA was thus subjected to PCR followed by agarose gel electrophoresis to probe the PCR efficiency. The presence of PCR bands demonstrated that cotton-based extraction was indeed capable of extracting gDNA from an aqueous solution with and without involving chaotropic salts (Lanes 3 and 4, Fig. 2A), with higher band intensity for that involving a chaotropic salt. Next, the extraction of *E. coli* gDNA from 30 % serum was investigated. As serum contains polymerase inhibitory heme, hormones, and immunoglobulin[1], a PCR involving gDNA template in serum would be partially or completely inhibited. If the direct or Gu-HCl-assisted extraction were successful, they would improve the PCR efficiency. Accordingly, the polymerase inhibiting nature of complex biofluid was evident from the presence of faint PCR band when the template DNA originated from 30 % serum (Lane 4, Fig. 2B). Both the chaotropic salt assisted as well as direct extraction then significantly helped improve the band intensity (Lanes 5 and 6, Fig. 2B), with greater efficiency for the former. Negative control experiments involved PCR performed on elution buffer (Lane 2, Fig.s 2A and B) or elution from a "direct extraction" workflow albeit without gDNA presence in the aqueous sample of origin (Lane 3, Fig. 2B). These experiments verified that the eluted gDNA did not originate from the cotton, toothpick, or elution buffer. Overall, these assays provided preliminary indication that toothpick mounted cotton could be an effective nucleic acid extraction tool and encouraged further experimentation involving biofluid composition and quantification of extraction efficiency.

Next, the possibility of extraction of *E. coli* gDNA from solutions containing a higher percentage (45 and 60 %) of serum was explored and compared with that of a 30 % serum-spiked solution. In principle, 45 and 60 % serum-spiked solution should contain an even greater quantity of polymerase inhibitory components and thus be expected to considerably inhibit PCR. The inhibiting nature of 45 and 60 % serum (in



Scheme 1. Nucleic acid extraction from complex biofluid using toothpick-actuated cotton. (A) Preparation of toothpick-mounted-cotton. (B) Extraction using chaotropic salt. (C) Direct extraction without using a chaotropic salt.

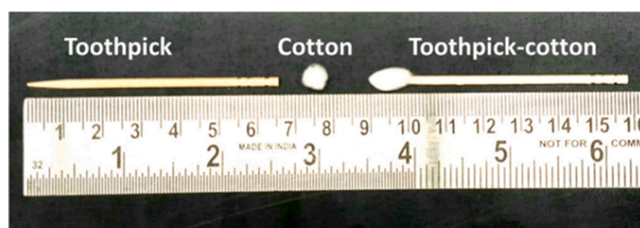


Fig. 1. Image of toothpick, cotton, and toothpick-mounted-cotton.

addition to that of 30 % serum) was again immediately apparent from the absence of a gel band for the PCR performed on gDNA spiked in these samples (Fig. 3A and B, Lanes 6–8). The desired band appeared in the PCR carried out on DNA extracted using chaotropic salt-assisted cotton-based purification (Fig. 3A, Lanes 3–5). Interestingly, the purification of DNA from these spiked samples was also effective using non-chaotropic salt-mediated direct extraction, albeit qualitatively less so (Fig. 3B, Lanes 3–5). Overall, this set of experiments qualitatively established that both Gu-HCl-assisted and direct extraction were able to "rescue" gDNA from 30 to 60 % serum, although with greater efficiency for the former method.

2.3. Extraction of DNA from 30 to 60 % serum and downstream qPCR

Next, the utility of cotton-based *E. coli* gDNA extraction was quantitatively probed using qPCR. Given the widespread adoption of qPCR in disease detection, its performance would primarily be reflective of how fruitful the extraction would be in routine detection-coupled-signal amplification processes in molecular diagnosis. When compared with the standard curve of absolute concentration vs cycle threshold value (C_t value, Fig. S1), it would also help quantify the efficiency of DNA recovery. Before assessing the impact of biofluid content on the extraction efficiency, we first probed if the amount of cotton may play a role in controlling the extraction efficiency. While difficult to investigate in a qualitative PCR-gel electrophoresis assay, this could be effectively quantified using qPCR analysis. Accordingly, the gDNA was spiked in 30 % serum and then subjected to direct and Gu-HCl-assisted extraction involving cotton amounts 2.5, 5.0, 7.5, or 10 mg. Next, both the gDNA template in 30 % serum or the same after its extraction into elution buffer was subjected to qPCR analysis (Fig. 4A). Due to PCR inhibiting nature of the serum, the gDNA template in it ("before extraction" samples in Fig. 4B and C) was anticipated to display a higher C_t value than its extracted counterpart (i.e., "after extraction" samples). Accordingly, the C_t values for "before extraction" samples were higher than that for "after extraction" samples (Fig. 4B and C). It also reflected 0.7–3.8 % (for Gu-HCl-assisted extraction) and 0.6–2.4 % (for direct extraction) gDNA

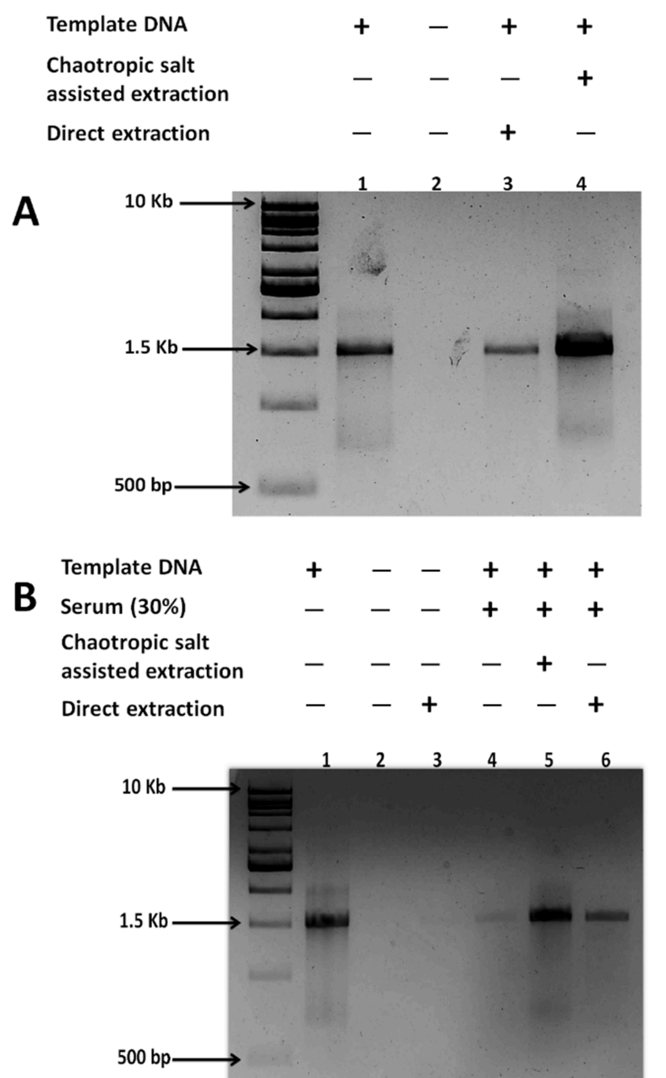


Fig. 2. Extraction of *E. coli* gDNA from aqueous solution and 30 % serum using toothpick-actuated cotton with and without chaotropic salt. (A) Extraction of *E. coli* gDNA from aqueous solution using toothpick-actuated cotton with and without chaotropic salt. (B) Extraction of *E. coli* gDNA from 30 % serum (v/v) using toothpick-actuated cotton with and without chaotropic salt. The positive control (lane 1, template in aqueous solution, no extraction) utilized 7.1 ng pure gDNA/reaction. Lane 2 in both panels are negative controls that involved PCR assays utilizing elution buffer.

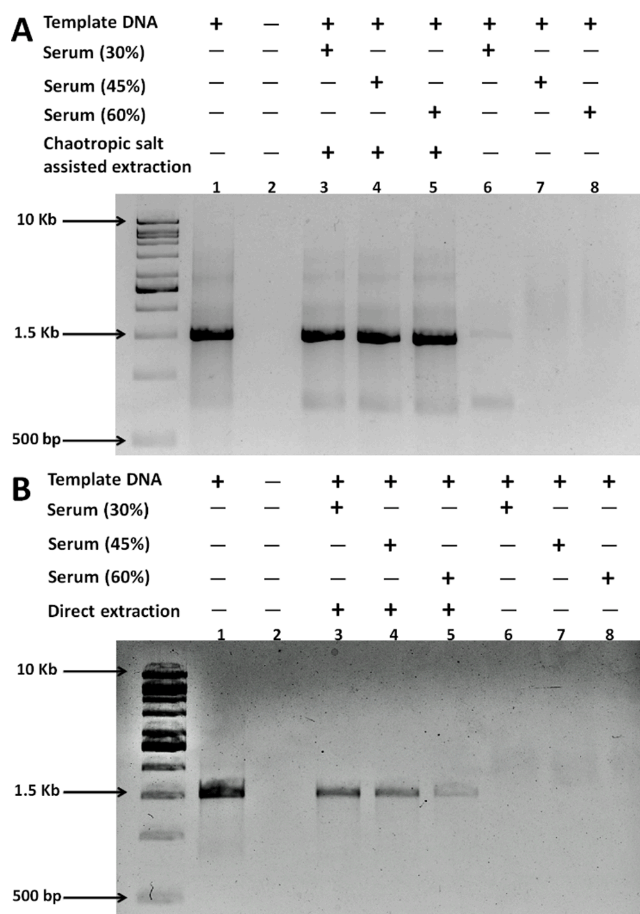


Fig. 3. Extraction of *E. coli* gDNA from 30, 45, and 60 % serum (v/v) using toothpick-actuated cotton with and without chaotropic salt. (A) Extraction of *E. coli* gDNA from 30, 45, and 60 % serum (v/v) using toothpick-actuated cotton with Gu-HCl as chaotropic salt. (B) Direct extraction of *E. coli* gDNA from 30, 45, and 60 % serum (v/v) using toothpick-actuated cotton without chaotropic salt. The positive control (lane 1, template in aqueous solution, no extraction) utilized 7.1 ng pure gDNA/reaction. Lane 2 in both panels were negative controls that involved PCR assays utilizing elution buffer.

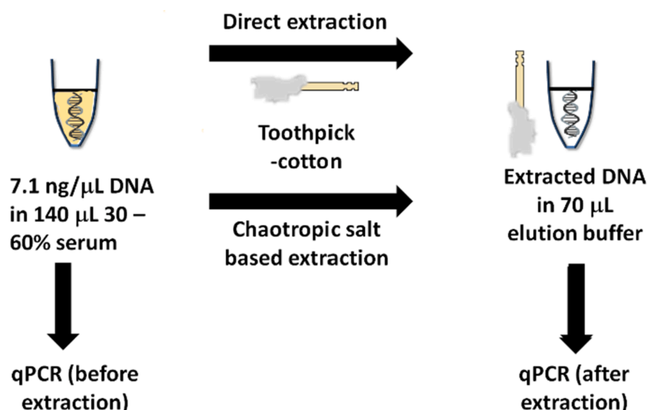
recovery (Table S2). However, there were no cotton amount-dependent trend in C_t values for "after extraction" samples, either in direct or Gu-HCl assisted extraction, despite 4-fold change in the cotton amount. This could be due to the fact the surface area of the cotton, where the nucleic acid presumably binds, did not change significantly over this range. It may require a greater amount of cotton, 10-fold or higher, to discern any statistically significant change. However, accommodation of such an amount of cotton would not be feasible in the 1.5 mL microcentrifuge tube of the current workflow and would require a larger tube along with a possibly higher amount of wash buffer and elution buffer as well.

To further probe the extraction performance, the C_t value for a positive control was compared with that of extracted (i.e., in elution buffer) as well as unextracted gDNA (i.e., latter present in 30, 45, and 60 % serum samples). The methodology of these experiments has been illustrated in Scheme 2 with the results summarized in Fig. 5, Fig. S2, and Table S2. The gDNA was spiked in 30, 45, or 60 % serum followed by direct or chaotropic salt-assisted extraction to elution buffer facilitated through toothpick-mounted cotton. Next, both the gDNA template present in the 30–60 % serum or elution was subjected to qPCR (i.e., "before extraction" samples). It was anticipated that the C_t value for gDNA template present in 30–60 % serum would be in similar range as that of NTC (i.e., qPCR carried out without template DNA) due to the serum's PCR inhibitory nature[1]. On the other hand, the C_t value would reduce when qPCR would be performed on the elution thanks to the removal of PCR inhibitory protein during the extraction procedure (i.e., "after extraction" samples).

Expectedly, the gDNA, when present in 30–45 % serum samples, demonstrated a high C_t value close to that of the NTC (i.e., 34–36, Fig. 5A and C, Fig. S2A–F). The C_t value considerably "improved" (i.e., reduced) for both chaotropic salt (to 24–26) as well as non-chaotropic salt (to ~ 28) assisted cotton-mediated extraction processes. When compared with a standard curve generated with a serially diluted pure gDNA sample (0.0071–7.1 ng/reaction, Fig. S1), this indicated 3.7–27.7 % DNA recovery in terms of C_t value of the extracted samples for chaotropic salt assisted extraction (Table S2). For direct extraction, the DNA recovery was in the range of 0.4–1.7 %. The high C_t value even after DNA extraction could of due to the continued presence of polymerase inhibitors (contamination carryover), poor DNA binding in the 1st step, loss of DNA during the wash steps, failure to release DNA at the elution step, or a combination of these factors.

Besides the bar plot representing C_t values of qPCR performed on

Fig. 4. Role of cotton amount on extraction efficiency in chaotropic salt (Gu-HCl) mediated (Panel B) or direct extraction (Panel C) of nucleic acid from 30 % serum. Panel A describes the schematics of experiment for assessing the influence of cotton amount in extraction efficiency. The "before extraction" samples in rest of experiments consisted gDNA in 30 % serum as qPCR template and are identical for all the experiments. Error bars represent standard deviation (n = 3) for biological replicates. The positive control (template in aqueous solution, no extraction) samples utilized 7.1 ng pure gDNA/reaction, where its corresponding NTC qPCR utilized elution buffer without any template gDNA. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001, ns; not significant.



Scheme 2. Method for assessing the qPCR-amenability of DNA extracted with or without using chaotropic salt.

gDNA before and after extraction, we also plotted this data using ΔC_t value, where $\Delta C_t = C_{t\text{-before extraction}} - C_{t\text{-after extraction}}$ (Fig. 5B and D). For a control experiment, $C_{t\text{-before extraction}}$ and $C_{t\text{-after extraction}}$ are to be obtained from qPCR performed on pure gDNA template in aqueous solution (i.e., without any extraction) and NTC with elution buffer instead of template, respectively. Accordingly, the ΔC_t value magnitude was the highest for the control qPCR experiments (Fig. 5B and D). For extraction experiments, the $C_{t\text{-before extraction}}$ would be from qPCR performed in gDNA template present in 30–45 % serum, while $C_{t\text{-after extraction}}$ was to be obtained from qPCR on gDNA extracted to elution buffer. In principle, ΔC_t therefore would be positive since the C_t value is anticipated to reduce after extraction. Furthermore, for a higher serum percentage (e.g., 60 % serum), the qPCR would be partially or completely inhibited, causing a high C_t value close to NTC performed with elution buffer. For a more effective extraction, the C_t value would considerably reduce, leading to a greater ΔC_t value magnitude. Therefore, ΔC_t magnitude would be reflective of the extraction efficiency. The comparison of ΔC_t value (the difference in C_t value from qPCR performed on extracted and unextracted gDNA) from 30, 45, 60 % serum-spiked samples further indicated that Gu-HCl based extraction performed better than direct extraction (Fig. 5B and D). The lesser ΔC_t for direct extraction (i.e., non-chaotropic salt mediated) (in the range of 3.7–6.9) compared to that of chaotropic salt mediated extraction (in the range of 5.9–10.6), especially for the higher serum percentage solutions, was indicative of possible contamination carryover in the earlier. Overall, it implied that a non-chaotropic salt mediated direct extraction would be less effective than Gu-HCl-assisted extraction in rescuing high quality gDNA from a biofluid sample.

In addition to guanidium hydrochloride-assisted and direct

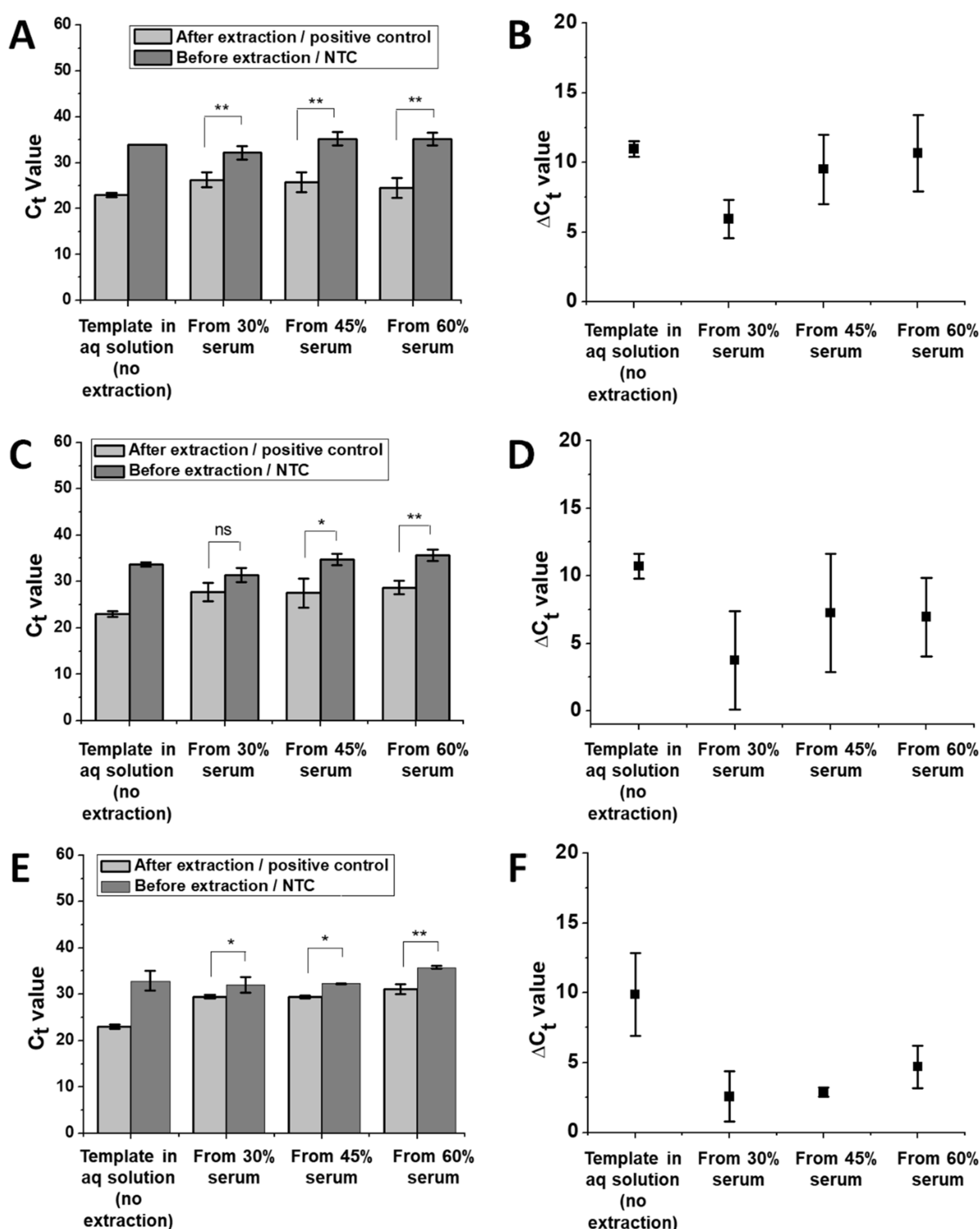


Fig. 5. qPCR-based quantification of E. coli gDNA extraction from 30, 45, 60 % using toothpick-actuated cotton. (A) Cycle threshold value (C_t value) for qPCR performed on gDNA before and after extraction from 30, 45, 60 % serum using toothpick-actuated cotton utilizing Gu-HCl as chaotropic salt. (B), ΔC_t value for Panel A. (C) C_t value for qPCR performed on gDNA before and after extraction from 30, 45, 60 % serum using toothpick-actuated cotton without utilizing chaotropic salt (i.e., direct extraction). (D), ΔC_t value for Panel C. (E) C_t value for qPCR performed on gDNA before and after extraction from 30, 45, 60 % serum using toothpick-actuated cotton with utilizing urea as chaotropic salt. (F), ΔC_t value for Panel E. Error bars represent standard deviation (n = 3) for biological replicates. ΔC_t represents $C_{t\text{-before extraction}} - C_{t\text{-after extraction}}$. The positive control (template in aq solution, no extraction) utilized 7.1 ng E. coli pure gDNA/reaction, where its corresponding NTC qPCR used elution buffer without template gDNA. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.

extraction, we also investigated urea-assisted extraction (Fig. 5E and F). Urea is a common denaturant used in denaturing DNA polyacrylamide gel electrophoresis as well as sometimes in protein gels[37,38]. Urea is neutral in pH 4–8, and thus would not be involved in a Gu-HCl-like salt-bridge formation between negatively charged DNA backbone and cellulose hydroxyl groups. Since it binds to DNA nucleobases via hydrogen bonding[39,40], we hypothesized that a similar interaction would take place with the cellulose hydroxyl group using a common urea molecule or a network of urea and water molecules. Such interaction would facilitate a successful DNA extraction from complex biofluid. Being a common fertilizer, urea is also more commonly available than guanidium hydrochloride. If successful, a urea-assisted extraction would thus be highly utilitarian in DNA extraction. For urea-assisted extraction, we again utilized the workflow described in Scheme 1B where the urea concentration was set at 4.5 M during the extraction step and wash steps, with the rest of the steps were unchanged. Similar to Gu-HCl and direct extraction (Scheme 2), we attempted to "rescue" *E. coli* gDNA spiked in 30–60 % serum that was anticipated to partially or completely inhibit qPCR. As a result, the high magnitude of C_t value before extraction would be expected to reduce after the extraction. Similarly, the ΔC_t magnitudes ($C_{t\text{-before extraction}} - C_{t\text{-after extraction}}$) were analyzed to gauge the extraction efficiency. We found that not only the C_t values were generally higher after extraction (in the range of 29–31), the ΔC_t (2.5–4.7) was less than both the other methods (Gu-HCl-assisted and direct extraction) (Fig. 5F). As explained earlier and shown for Gu-HCl, higher the ΔC_t magnitude, better the extraction efficiency. But for urea, the ΔC_t magnitude was quite low (Fig. 5F), implying the "before extraction" (i.e., when qPCR gDNA template was in serum) and "after extraction" (i.e., when gDNA was extracted to elution buffer) was not too different. This held true for all three serum compositions investigated (Fig. 5E). It thus indicated a poor extraction efficiency. When compared to C_t value vs DNA amount (Fig. S1), it also indicated that the inefficiency of DNA loss was in the range of three orders of magnitude (0.03–0.2 % recovery, Table S2). It could be possible that urea, contrary to our hypothesis, did not form a bridging H-bond network between DNA and cellulose. Additionally, the poor qPCR performance could also be due to the retention of some urea molecules in the elution that may have inhibited the DNA polymerase activity[41]. Accordingly, agarose gel-

based analysis or sequencing (below) was not performed for urea-extracted DNA samples.

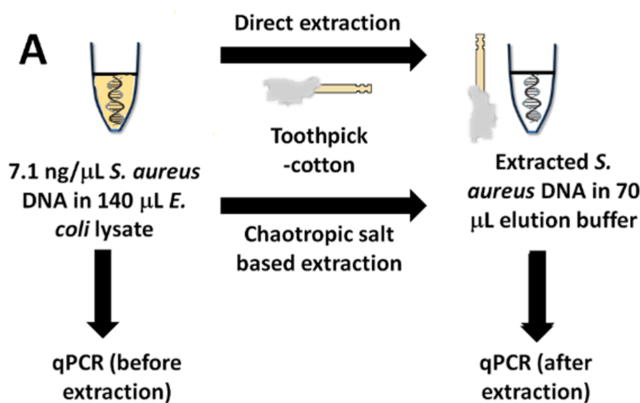
Beside the clinically important nucleic acid extraction from biofluids such as serum and plasma, another routine application of nucleic acid extraction remains in the DNA purification from cell lysate. It is frequently performed in academic and industrial labs using spin columns and high-speed centrifuges. If successful, cotton-based instrument-free extraction from cell lysate would be highly effective as an inexpensive yet rapid method. To investigate, we spiked *S. aureus* gDNA in *E. coli* lysate, subjected it to direct and Gu-HCl-assisted extraction, and then analyzed both the lysate-spiked as well as extracted (i.e., in elution) gDNA with qPCR (Fig. 6A). The lysate contained sodium dodecyl sulfate (SDS) that could cause PCR inhibition even in quantity as low as 0.05 % SDS[42]. Therefore, a reduction in C_t value from "before extraction" (i.e., gDNA in lysate) to "after extraction" (i.e., gDNA in elution) would be testimonial of the extraction utility. In this case, *S. aureus* gDNA was employed as the extraction target since exact quantities of the same could be quantitatively added to the *E. coli* lysate that would already contain unspecified amount of *E. coli* gDNA. Additionally, due to the orthogonal nature of *S. aureus* primers (against *vraR* gene) with respect to that of *E. coli* gDNA, non-specific amplifications would be avoided. Interestingly, the qPCR performed in *S. aureus* gDNA in lysate (i.e., "before extraction") did not demonstrate a characteristic amplification curve (hence a "blank" bar plot), reaffirming the strong PCR inhibitory nature of SDS (Fig. 6B and S3). After both the direct as well as Gu-HCl-assisted extraction, the characteristic "S"-shaped amplification profile was recorded, although with a higher C_t value than that of the positive control (i.e., qPCR involving pure *S. aureus* gDNA template). Although a more quantitative estimation of extraction efficiency was not performed, this experiment qualitatively established that both the direct as well as Gu-HCl-assisted extraction would be useful in extracting gDNA from cell lysate.

2.4. Compatibility with sequencing analysis

In addition to qPCR as a quantitative diagnostic method, sequencing analysis has been utilized for identifying single nucleotide variants as well as insertions and deletions, identification of large structural variants, and quality control through genotype screening among others[43]. Besides identifying genetic diseases and applications in agricultural biotechnology, such methods have recently been extensively used for detecting variants during pandemics such as SARS-CoV-2[44]. However, the quality of sequencing data is dependent on extracted gDNA or RNA on which the amplification using fluorescent dNTPs were to be performed. To see the compatibility of the cotton-based extraction methods with sequencing, the extracted DNA samples were subjected to PCR and then analyzed for Sanger sequencing. If the quality of extracted gDNA would be poor, it would be reflected in the resulting PCR and subsequently in the sequencing analysis. The result and its analysis

Table 1
Sequencing analysis performed on extracted gDNA through direct (without chaotropic salt) or Gu-HCl-based toothpick-cotton method.

Sample ID	Related bacterial strain	Similarity %
Positive control (PCR performed on pure gDNA)	*E. coli* strain HCD22-4 16S ribosomal RNA gene, partial sequence	94.79 %
	E. coli strain ISO3 16S ribosomal RNA gene partial sequence	94.31 %
	E. coli strain ECIUP_15 16S ribosomal RNA gene, partial sequence	94.25 %
Direct extraction from 30 % serum	*E. coli* strain TS 16S ribosomal RNA gene, partial sequence	83.18 %
	E. coli voucher ST401 16S ribosomal RNA gene, partial sequence	83.17 %
	Uncultured bacterium clone PB146 16S ribosomal RNA gene, partial sequence	83.15 %
Direct extraction from 45 % serum	*E. fergusonii* strain RJF32_Dcrust2 16S ribosomal RNA gene, partial sequence	74.51 %
	E. coli strain Huaian_106_3 16S ribosomal RNA gene, partial sequence	74.51 %
	E. coli strain E2 chromosome, complete genome	74.50 %
Direct extraction from 60 % serum	Did not yield any match	
Direct extraction from 30 % serum (with Gu-HCl)	*E. coli* strain VRR-1B 16S ribosomal RNA gene, partial sequence	98.23 %
	E. coli strain M10SaCRM5 16S ribosomal RNA gene, partial sequence	98.13 %
	E. coli strain 212 16S ribosomal RNA gene, partial sequence	98.13 %
Direct extraction from 45 % serum (with Gu-HCl)	*E. coli* strain HILLA-1 16S ribosomal RNA gene, partial sequence	97.74 %
	E. coli strain RN122 16S ribosomal RNA gene, partial sequence	97.74 %
	E. coli strain RI33 16S ribosomal RNA gene, partial sequence	97.72 %
Direct extraction from 60 % serum (with Gu-HCl)	*E. coli* partial 16S rRNA gene, isolate OPB4	86.05 %
	E. coli strain EGE 4471157–64 16S ribosomal RNA gene, partial sequence	85.13 %
	E. coli strain CEE7 16S ribosomal RNA gene, partial sequence	84.72 %



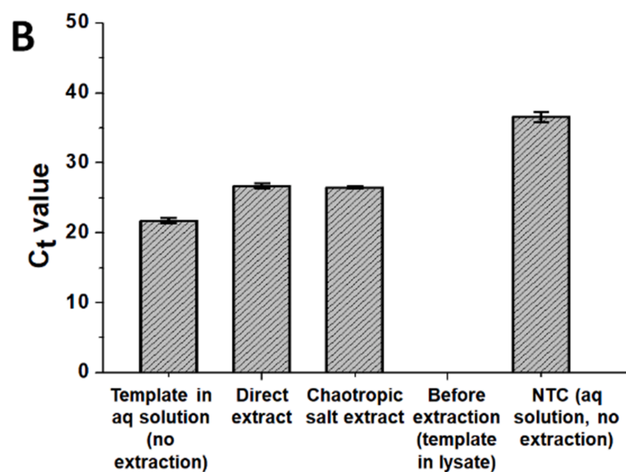


Fig. 6. qPCR compatibility assessment of *S. aureus* gDNA extracted from E. coli lysate. (A), Schematics of experiment. (B), qPCR suitability of *S. aureus* gDNA extracted using direct extraction or chaotropic salt mediated extraction. The positive control (template in aqueous solution, no extraction) utilized 7.1 ng *S. aureus* pure gDNA/reaction, where its corresponding NTC qPCR used elution buffer without template gDNA.

through NCBI BLAST for sequence identity determination of the amplicon are present in Table 1. The results indicated a high degree of similarity (~85–98 %) achieved with Gu-HCl-assisted extraction with maximum identity with the partial sequence of 16S rRNA gene from *E. coli*, the target gene of the PCR in this study. For direct extraction from 30 to 45 % serum, the similarity score was relatively lower (~74–83 %) compared to its Gu-HCl-assisted counterpart and in consonance with poorer PCR gel band intensity and qPCR efficiency seen above. The high similarity score obtained for Gu-HCl-based method therefore implied that DNA extracted using it would be compatible with Sanger sequencing, i.e., polymerase amplification with fluorescent nucleotide insertion.

To better understand the inhibitory protein removal during the extraction, we performed direct and Gu-HCl-assisted "mock" nucleic acid extraction on 60 % serum solution, but one devoid of any spiked nucleic acid (Fig. S4A). The elution was then quantified for protein presence using A280. Post-extraction, the protein amount in 60 % serum (19,651 ng/μL) reduced to 4,433 ng/μL (8.8-fold decrease) for direct extraction and 128 ng/μL (306-fold decrease) for Gu-HCl-assisted extraction (fold change calculated after accounting for 50 % decrease in elution buffer volume compared to sample of origin) (Fig. S4B). Despite the 8.8-fold protein removal after direct extraction from 60 % serum, the still significant protein presence in the elution would presumably be responsible for poor performance in PCR, qPCR and sequencing. In comparison, the negligible protein presence after Gu-HCl-assisted extraction was favourable for the downstream NAAT assays.

To understand the nature of the interaction between the nucleic acids and the medical-grade cotton and the plausible reason behind the enhanced nucleic acid extraction efficiency by the cotton in presence of chaotropic salt Gu-HCl, the cotton's surface charge (measured through the zeta potential) could be utilized. We found that the surface of medical-grade cotton has a negative zeta potential which mostly arises from the presence of hydroxyl groups on cellulose[45] (Fig. 7). The zeta potential decreases with increasing pH (pH 2 to pH 8) which can be attributed to the swelling of the surface layers of the cotton fiber. Easier access of anionic groups upon swelling possibly causes the decrease in zeta potential[45]. Nucleic acid carries a net negative charge, largely due to the presence of an electronegative phosphate backbone, the like charges between the DNA and the cotton surface result in a repulsive force that may hinder the DNA binding and relatively lesser efficiency for direct extraction. The addition of chaotropic salts, such as guanidium hydrochloride (Gu-HCl) increases the binding of DNA to the cotton by counteracting the electrostatic repulsion and hence leading to an efficient DNA extraction compared to direct extraction. Moreover, the negative zeta potential in an acidic pH (pH 4–5) would in principle enable RNA extraction as well. This would possibly lead to several important applications such as mRNA as well as other cellular RNA purification and viral RNA extraction. This would be taken up in future applications.

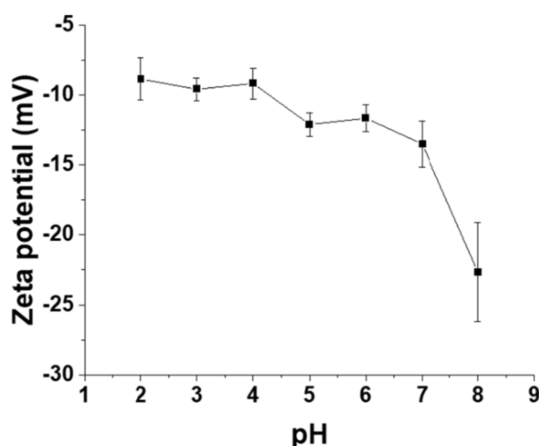


Fig. 7. Zeta potential measurement of cotton suspension as a function of pH.

2.5. *Comparison with published studies, cost, limitation, scope of applications, and future experiments*

Next, we compared the DNA extraction using toothpick-mounted cotton with other published methods of minimal instrument intensive nucleic acid purification summarized in Table S1. To the best of our knowledge, this method probably would be the only one that utilizes readily available over-the-counter and household materials for rapid (10–30 min) and NAAT-compatible nucleic acid extraction. Besides requiring minimal "fabrication time" (mounting and sterilization), it would also be extremely low cost (less than INR 10 or $0.12) in terms of raw material cost and lower than most of the methods listed in Table S1. While this preliminary study successfully demonstrates that toothpick-actuated cotton would be capable of extracting DNA from complex biofluid, the extraction efficiency remained low. Also, the semi-quantitative nature of this study made it difficult to identify the precise reason behind the loss of inefficiency. Numerous factors such as incubation time in the capture, wash, or elution steps, salt concentration, solution pH, cotton amount, and immersion frequency could influence the extraction efficiency. Our future studies would probe the role of these factors in improving extraction efficiency. We would also investigate DNA extraction from other complex biofluids as well as food and beverages, from plant cell lysates, detergent compatibility, and most importantly application in molecular diagnosis for clinical samples. Similarly, the possibility of automated or manual extraction devices fitted with cotton would be looked into. The inefficient extraction at the current stage would probably limit its application for qPCR-based pathogen nucleic acid detection and accurate load estimation from a patient sample. However, the method would still permit other applications such as sample preparation for sequencing-based genetic disease screening and extraction-amplification of DNA from an environmental source.

3. Conclusion

This study aims to investigate the toothpick-actuated cotton for its ability to extract DNA from complex biofluid solutions with or without using chaotropic salt. To investigate, DNA spiked in 30, 45, and 60 % serum was extracted using toothpick-cotton in the presence and absence of Gu-HCl, where the latter procedure was termed as direct extraction. The extracted nucleic acid was subjected to PCR-gel electrophoresis, qPCR, and DNA sequencing. As evident by the presence of desired bands in gel, improved (reduced) C_t value after extraction, and sequence identity match in DNA sequencing, both procedures yielded DNA extraction, albeit with low recovery and different efficiencies. When Gu-HCl was utilized as the chaotropic salt, it provided relatively higher efficiency compared to direct extraction due to significantly more effective removal of polymerase inhibitors as well as better DNA binding to cotton. Additionally, both the Gu-HCl-assisted and direct extraction were able to "rescue" qPCR-compatible gDNA present in cell lysate containing polymerase inhibitory SDS. On the other hand, extraction using urea, another chaotropic salt performed worse than even direct extraction. Overall, we anticipate that this study will initiate a closer look into the ability of cotton and cotton-fitted devices in DNA extraction at limited-resource and near-point-of-care disease detection.

4. Materials and methods

Trizma base (#93362), EDTA-Na_2 (#E5134), Ethyl alcohol (CAS # 64–17-5) were purchased from Sigma-Aldrich, India while the rest of the chemicals were purchased from SRL Chemicals, India unless mentioned otherwise. 2 × real-time SYBR master mix (# MBT074) and 2 × PCR

TaqMixture (# MBT061) were procured from HiMedia, India. 1.5 mL of centrifuge tubes was from Tarsons, India. Primer oligonucleotides (desalting purified) were purchased from Eurofins. Additionally, medical-grade cotton and regular-use toothpick were purchased from the local medical shop. DNA concentration estimations using UV_{260} were carried out at the Thermo MultiSkan Go plate reader. Real-time PCR experiments were carried out in BioRad CFX Maestro or Connect instrument. PCR and gel visualization was carried out at the Eppendorf master cycler and Bio-Rad ChemiDoc™ respectively. The zeta potential measurements were conducted at UGC-DAE Consortium for Research, Kolkata. The sequencing analysis was carried out at Barcode Biosciences, India.

4.1. Nucleic acid spiking

The nucleic acid spiking was tested with a bacterial genomic DNA (gDNA, obtained from *Escherichia coli* (*E. coli*) DH5α strain) spiked in aqueous or with three different concentrations of 30 %, 45 %, and 60 % (v/v) of fetal bovine serum (FBS, Gibco) samples in aqueous solution. In each case, the assay was performed on 7.1 ng/μL in 140 μL solution. For details on *S. aureus* gDNA spiking in *E. coli* lysate, please see Section 4.6.

4.2. Nucleic acid extraction using chaotropic salt with Gu-HCl

The gDNA extraction-amplification assay was tested with three different concentrations (final 30 %, 45 %, and 60 %, v/v) of FBS spiked gDNA or 1 × TE buffer. In each case, the assays were performed with 7.1 ng/μL of gDNA in 140 μL of solution. For chaotropic salt-assisted extraction, the aqueous solution or the serum-spiked solution containing the gDNA was first added with 140 μL of 2 × lysis buffer (4 M Gu-HCl, 100 mM Tris-HCl pH 7.5). DNA capture was carried out by using 5–5.5 mg of cotton that was dipped into the 280 μL biofluid-lysis buffer mix with the help of a toothpick, followed by 5 min benchtop incubation with occasional twirling (2 times per min). After four successive washing, including two washes with 400 μL of wash buffer (60 % isopropanol and 2 M Gu-HCl, 2 min incubation) and two washes with 300 μL of 80 % ethyl alcohol (2 min incubation), the toothpick-cotton was left for air drying. After 10 min of air-drying, bound DNA was eluted in 70 μL of elution buffer (10 mM Tris-HCl buffer pH 8, 5 min incubation). 1 μL of the elution was subjected to real-time PCR.

4.3. Nucleic acid extraction using chaotropic salt with urea

The gDNA extraction and amplification assay with urea as a chaotropic salt are similar to the Gu-HCl method (section 4.2), with the gDNA was spiked with 30 %, 45 %, and 60 %, (final, v/v) FBS or 1 × TE buffer. The assays were performed with 7.1 ng/μL of DNA in 140 μL of biofluids. The aqueous solution or the serum was added with 140 μL of 2 × lysis buffer (9 M Urea, 100 mM Tris-HCl pH 7.5). DNA was captured using 5–5.5 mg of cotton that was dipped in the 280 μL lysis buffer-biofluid mix with the help of a toothpick, followed by 5 min benchtop incubation with occasional twirling (2 times per min). After four successive washing, including two washes with 400 μL of wash buffer (60 % isopropanol and 4.5 M urea, 2 min incubation) and two washes with 300 μL of 80 % ethyl alcohol (2 min incubation). After 10 min of air drying the toothpick-cotton, bound DNA was eluted in 70 μL of elution buffer (10 mM Tris-HCl buffer pH 8, 5 min incubation). 1 μL of the elution was subjected to real-time PCR.

4.4. Nucleic acid extraction without using chaotropic salt (direct extraction)

Without using chaotropic salt, the DNA extraction method was performed with the same FBS spiked gDNA (final 30 %, 45 %, and 60 %, v/v) or 1 × TE buffer. The initial gDNA concentration was 7.1 ng/μL in 140 μL of aqueous solution or bio-fluid. The DNA extraction was done by dipping 5–5.5 mg of cotton with a toothpick and left for 5 min of benchtop incubation with occasional twirling (2 times per min). Finally, the bound DNA was eluted in 70 μL of elution buffer (without drying, 5 min incubation) and 1 μL of the elution was used for real-time PCR.

4.5. Assessment of the impact of cotton amount on the extraction efficiency

For direct and Gu-HCl assisted extraction as described above, we utilized cotton amounts 2.5, 5.0, 7.5, or 10.0 mg. Rest of the parameters and time durations were kept identical as that of the extraction workflow described in Sections 4.2 and 4.4. 1 μL of the elution was subjected to real-time PCR analysis.

4.6. Extraction of S. aureus gDNA from E. coli cell lysate

An overnight grown 5 mL *E. coli* (DH5α) culture (O.D. ~ 1) was pelletized (3500 r.c.f., 15 min), washed with PBS, resuspended and treated with 1 mL lysis buffer (final composition Tris 10 mM, EDTA 5 mM, SDS 0.5 % (w/v), proteinase K 100 μg/mL) at 50 °C for 12–18 h. Next, the solution was centrifuged (12000 r.c.f., 15 min) and 120 μL of the supernatant was collected. The same was then mixed with 10 μL TE buffer and 10 μL *S. aureus* gDNA (stock concentration 100 ng/μL in TE buffer), leading to 7.1 ng/μL *S. aureus* gDNA in 140 μL *E. coli* cell lysate solution. This was subjected to direct or Gu-HCl assisted extraction exactly as described above in Sections 4.2 and 4.4 (with 5.0–5.5 mg cotton) and 1 μL of the elution was used for real-time PCR.

4.7. Quantification of protein presence in elution

140 μL 60 % serum solution was subjected to direct or Gu-HCl assisted extraction exactly as described above in sections 4.2 and 4.4 (with 5.0–5.5 mg cotton). The elutions were subjected to A280 measurement in a Thermo MultiSkan Go plate reader.

4.8. Method of PCR and gel electrophoresis analysis

To assess DNA quality, PCR amplification was performed. The reaction was performed in a 15 μL volume, where the template was the 1 μL DNA in spiked solution or 1 μL elution from the above extraction methods. The elution was added with 2 × Taq mastermix (7.5 μL), forward (5′-AGAGTTTGATCATGGCTCAG-3′) and reverse primers (5′-GGTTACCTTGTTACGACTT-3′) (final concentration 0.3 μM), and molecular-grade water. The primers were against the *E. coli* 16S rRNA gene (Accession no: NZ_CP080399.1). For amplification, the cycle was set at 95 °C for 180 s, then 45 cycles of 95 °C for 10 s, 52 °C for 10 s, and 72 °C for 30 s, where the last step includes the amplification analysis. The amplicons were resolved on 1 % agarose gel at 70 V with a 1 Kb ladder. The electrophoretogram was observed by using the ChemiDoc™ XRS + Imaging System (Bio-Rad, Hercules, CA, USA).

4.9. Method of qPCR

To check the quantitative real-time PCR (qPCR) compatibility, a 15 μL reaction of reaction was carried out containing 1 μL DNA in spiked solution or 1 μL elution from the above extraction methods. For the *S. aureus* gDNA amplification, forward (5′-CCATGAGTTGAAGCCA-GATTT-3′) and reverse (5′-CCTGCATCTAATGCACGATA-3′) primers against *vraR* gene were used. This was mixed with 2 × real-time Taq PCR mix (7.5 μL), forward and reverse primer (same as above, final concentration 0.3 μM), and molecular biology grade water. The thermal program was set at 95 °C for 180 s, then 45 cycles of 95 °C for 10 s, 52 °C for 10 s, and 72 °C for 30 s. The last step was fluorescence monitoring followed by a default program of melt curve analysis.

4.10. Sequencing and data analysis

For sequencing, PCR was performed in 30 μL of reaction volume where the template (2 μL elution from above extractions) that was added with 2 × Taq mastermix (without SYBR, 15 μL), forward and reverse *E. coli* primers (same as above, final concentration 0.3 μM), and molecular biology grade water. For amplification, the program was at 95 °C for 180 s, then 45 cycles of 95 °C for 10 s, 52 °C for 10 s, and 72 °C for 30 s. PCR products were further purified by PCR purification kit (HiGenoMB, HiMedia), and then sent for Sanger sequencing (3730xl DNA Analyzer at an external facility). The sequencing data was viewed through FinchTV software (Geospiza, Inc.). The sequence was analyzed through BLAST to determine the sequence identity of the amplicon.

4.11. Sample preparation and method of zeta potential measurement

Zeta potential measurement was carried out to measure the charge on the cotton fiber at different pH values (pH 2–8). The cotton fiber was finely cut into tiny pieces (of approximately 1 mm length) and suspended (final concentration 0.1 %, w/v) into 100 μL of citrate–phosphate buffers of different pH (2–8). The samples were equilibrated by vibrating for 5 min before the measurement. The zeta potential was estimated at 25 ± 0.1 °C by using the commercial Zetasizer Nano ZS from Malvern Panalytical ltd, UK and the measurement was repeated 3 times.

Data availability

The raw data files for this study, video, and an index for the same have been uploaded in Zenodo with the https://doi.org/10.5281/zenodo.6412122.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data has been shared at Zenodo repository

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Appendix A. Supplementary material

The supplementary data contains Tables summarizing existing literature in this field and recovery efficiency, qPCR analysis for C_t value vs DNA (in ng and copy numbers), representative amplification plots for extraction experiments, and protein quantification post-extraction. Two video files showing chaotropic salt-assisted extraction and direct extraction have been provided. Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmc.2022.117009.

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