Supplementary Information (SI) for Lab on a Chip.


**Supplementary material file**

Table S1. *Staphylococcus aureus* reference strains included in this study. Details of the enterotoxin genes carried by each reference strain are provided, along with the origin of each strain (sample type and geographic origin) where available.

| Strain | Enterotoxin genes carried | Source of isolate (sample type) and country of origin |
|---|---|---|
| DSMZ 19041 | A, B, D, G, I, J, Panton Valentine Leucocidin | human furuncle, Czech Republic |
| DSMZ 19044 | B, G, I | human pus, Czech Republic |
| DSMZ 20652 | B, G, I | Not provided |

*DSMZ= Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures.

Table S2. Loop-mediated isothermal amplification (LAMP) primer sets used for detection of *Staphylococcus aureus* enterotoxin A and B genes (SEA and SEB).

| Target | Primer | 5'–3' Oligonucleotide sequence* | Reference |
|---|---|---|---|
| SEA | Forward inner primer (FIP) | CCT GTAAATAACGTCTTGCTTGAAGA TAC AGT ACC TTT GGA AAC G (F1c-F2) | This study |
| | Backward inner primer (BIP) | CTG ATG TTT TTG ATG GGA AGG TTC CCG AAG GTT CTG TAG AAG T (B1-B2c) | (Goto et al. 2007) |
| | Forward primer (F3) | TCA ATT TAT GGC TAG ACG GT | |
| | Backward primer (B3) | CTT GAG CAC CAA ATA AAT CG | |
| | Loop forward primer (LF) | GTAACTGTTCAGGAGTTGG (LF) | |
| | Loop backward primer (LB) | AGA GGG GAT TAA TCG TGT TTC A | (Goto et al. 2007) |
| SEA (tagged) (LF and LB primers) | FIP | GAT CCA ACT CCT GAA CAG TTA CAA TAC AGT ACC TTT GGA AAC G (F1c-F2) | |
| | BIP | CTG ATG TTT TTG ATG GGA AGG TTC CCG AAG GTT CTG TAG AAG T (B1-B2c) | |
| | F3 | TCA ATT TAT GGC TAG ACG GT | |
| | B3 | CTT GAG CAC CAA ATA AAT CG | |
| | LF | **FITC-** GTAACTGTTCAGGAGTTGG | |
| | LB | **Biotin**- AGA GGG GAT TAA TCG TGT TTC A | (Goto et al. 2007) |
| SEB | FIP | CAC CAA ATA GTG ACG AGT TAG GTA AGA CGT ACA AAC TAA TAA GAA AAA GG (F1c-F2) | This study |
| | BIP | ACT CTA TGA ATT TAA CAA CTC GCC TTG TCA TAC CAA AAG CTA TTC TCA (B1-B2c) | |
| | F3 | GTT CGG GTA TTT GAA GAT GG | |
| | B3 | TTG GTC AAA TTT ATC TCC TGG | |
| | LF | TCT AAT TCT TGA GCA GTC A | |
| | LB | ATG AAA CGG GAT ATA TTA AAT TTA T | |

| | | | |
|---|---|---|---|
| SEB (tagged) (LF and LB primers) | FIP | CAC CAA ATA GTG ACG AGT TAG GTA AGA CGT ACA AAC TAA TAA GAA AAA GG (F1c-F2) | |
| | BIP | ACT CTA TGA ATT TAA CAA CTC GCC TTG TCA TAC CAA AAG CTA TTC TCA (B1-B2c) | |
| | F3 | GTT CGG GTA TTT GAA GAT GG | |
| | B3 | TTG GTC AAA TTT ATC TCC TGG | |
| | LF | **FITC-** TCT AAT TCT TGA GCA GTC A | |
| | LB | **Biotin**- ATG AAA CGG GAT ATA TTA AAT TTA T | |

*The text in red indicates the sequences of the newly designed SEA LAMP primers. The green and yellow highlighting shows the labels (tagging) of primers. The blue highlights show the different parts of the forward and backward inner primers.

Table S3. Manually designed artificial oligonucleotide sequences for *Staphylococcus aureus* enterotoxin A and B genes (SEA and SEB) for evaluation of loop-mediated isothermal amplification (LAMP) primers.

| Gene | Oligonucleotide Sequence | Size (nucleotides) |
|---|---|---|
| SEA gene | AAAAGTGCCAATCAATTTATGGCTAGACGGTAAACAAAATACAGTACCTTT GGAAACGGTTAAAACGAATAAGAAAAATGTAACTGTTCAGGAGTTGGATC TTCAAGCAAGACGTTATTTACAGGAAAAATATAATTTATATAACTCTGATG TTTTTGATGGGAAGGTTCAGAGGGGATTAATCGTGTTTCATCTTCTACAG AACCTTCGGTTAATTACGATTTA TTTGGTGCTCAAGGACAGAATTC | 249 |
| SEB gene | TAGAAGTATTACTGTTCGGGTATTTGAAGATGGTAAAAATTTATTATCTTTT GACGTACAAACTAATAAGAAAAAGGTGACTGCTCAAGAATTAGATTACCT AACTCGTCACTATTTGGTGAAAAATAAAAAACTCTATGAATTTAACAACTC GCCTTATGAAACGGGATATATTAAATTTATAGAAAATGAGAATAGCTTTTG GTATGACATGATGCCTGCACCAGGAGATAAATTTGACCAATCTAAATATTT AATGA | 260 |

Table S4. Primer sequences used for molecular confirmation of the presence of enterotoxin genes A and B (SEA and SEB) in the *Staphylococcus aureus* reference strains.

| Gene | primer | Sequence (5'-3') | Size of amplified product (bp) | Accession number | Reference |
|---|---|---|---|---|---|
| SEA | Forward | GGTTATCAATGTGCGGGTGG | 102 | M18970 | (Mehrotra et al. 2000; Yin et al. 2016) |
| | Reverse | CGGCACTTTTTTCTCTTCGG | | | |
| SEB | Forward | GTATGGTGGTGTAACTGAGC | 164 | M11118 | (Mehrotra et al. 2000; Yin et al. 2016) |
| | Reverse | CCAAATAGTGACGAGTTAGG | | | |

Table S5. Limit of detection (LOD) for *Staphylococcus aureus* enterotoxin (SE) genes using loop-mediated isothermal amplification (LAMP) at 60 °C for 30 minutes in thermocycler.

| (CFU/ml) | LAMP for detection of SEA (*S. aureus* DSMZ 19041 reference strain) | | | |
|---|---|---|---|---|
| | Cow | | Goat | |
| | No heating | Heating | No heating | Heating |
| LOD | $10^4$ | $10^4$ | $10^4$ | $10^3$ |
| | LAMP for detection of SEB (*S. aureus* DSMZ 19041, 19044 and 20652 reference strains) | | | |
| LOD | $10^4$ | $10^3$ | $10^4$ | $10^3$ |
| | Multiplex LAMP for detection of SEA and SEB (*S. aureus* DSMZ 19041 reference strain) | | | |
| LOD | $10^5$ | $10^4$ | $10^5$ | $10^4$ |

Table S6. Limit of detection (LOD) for *Staphylococcus aureus* enterotoxin (SE) genes using loop-mediated isothermal amplification (LAMP) at 60 °C for 60 minutes, in thermocycler.

| | Goat milk samples spiked with *S. aureus* DSMZ 19041 reference strain (CFU/ml) |
|---|---|
| SEA | $10^3$ |
| SEB | $10^3$ |
| SEA & SEB (Duplex) | $10^3$ |

Table S7. Statistical analysis using a two-way ANOVA of amplification data of loop-mediated isothermal amplification (LAMP) and multiplex LAMP assays for detection of *Staphylococcus aureus* enterotoxin A and B (SEA and SEB) genes. P-values < 0.05 were considered significant (*). Based on assays for 3 biological samples and 3 technical replicates for each.

| Statistical difference between amplification times (minutes) of LAMP assay for | P value |
|---|---|
| DNA extracts from spiked, diluted milk samples versus non-extracted spiked diluted milk samples | <0.001* |
| Detection of *S. aureus* SEA in heated versus unheated spiked diluted cow milk samples | <0.001* |
| Detection of *S. aureus* SEA in heated versus unheated spiked diluted goat milk samples | 0.034* |
| Detection of *S. aureus* SEB in heated versus unheated spiked diluted cow milk samples | 0.540 |
| Detection of *S. aureus* SEB in heated versus unheated spiked diluted goat milk samples | 0.033* |
| Multiplex LAMP assay for detection of *S. aureus* SEA in heated versus unheated spiked diluted cow milk samples | <0.001* |
| Multiplex LAMP assay for detection of *S. aureus* SEA in heated versus unheated spiked diluted goat milk samples | <0.001* |
| Detection of *S. aureus* SEB in heated versus unheated spiked diluted cow milk samples | <0.001* |
| Detection of *S. aureus* SEB in heated versus unheated spiked diluted goat milk samples | <0.001* |
| Singleplex versus multiplex LAMP assay for detection of *S. aureus* SEA and SEB in unheated (no 95 °C lysis pre-treatment) spiked diluted goat milk samples after running assays for 60 minutes | 0.841 |

Table S8. Limit of detection (LOD) of loop-mediated isothermal amplification (LAMP) and multiplex LAMP assay for *Staphylococcus aureus* enterotoxin A and B genes (SEA and SEB) genes in spiked 1:10 diluted goat milk samples at 60 °C incubation on a heat block for 30, 45 and 60 minutes.

| Colony forming units/ml (CFU/ml) | | Time (minutes) | | |
|---|---|---|---|---|
| | | 30 | 45 | 60 |
| | | SEA | | |
| LOD (*S. aureus* DSMZ 19041) | | $10^4$ | $10^4$ | $10^3$ |
| | | SEB | | |
| LOD (*S. aureus* DSMZ 19041) | | $10^5$ | $10^4$ | $10^3$ |
| | | Multiplex | | |
| LOD | *S. aureus* DSMZ 19041 | $10^5$ | $10^4$ | $10^3$ |
| | *S. aureus* DSMZ 19044 | $10^5$ | $10^4$ | $10^3$ |
| | *S. aureus* DSMZ 20652 | $10^5$ | $10^4$ | $10^3$ |

Table S9. Biochemical test results for the suspected colonies grown on sheep blood agar from two milk samples (field collected); both showed Gram positive cocci.

| Tests | Sample (L1) | Sample (L3) (Positive device) | Expected results of *Staphylococcus aureus* |
|---|---|---|---|
| Coagulase | - | - | + |
| Nitrate | + | + | + |
| MIO (Motility Indole Ornithine Tests) | -,-,- | -,-,- | -,-,- |
| Urease | - | - | + |
| Mannitol | - | - | + |
| Maltose | + | - | + |
| Lactose | + | + | + |
| Xylose | - | - | - |
| Sucrose | + | + | + |
| Sorbitol | - | - | - |
| Novobiocine | Susceptible | Susceptible | Susceptible |
| Bacitracin | Susceptible | Susceptible | Susceptible |
| Polymyxin B | Susceptible | Susceptible | Resistant |
| Furazolidone | Susceptible | Susceptible | |

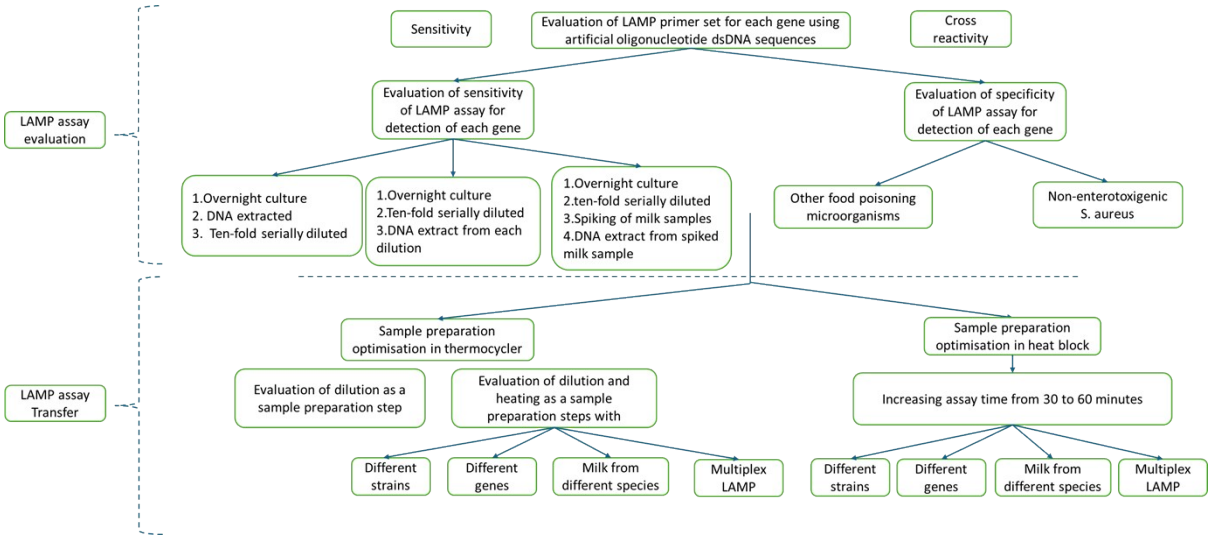


Figure S1. Diagram outlining the different steps taken in the evaluation, optimisation and transfer of Loop-mediated isothermal amplification (LAMP) assays (moving from being performed in a thermocycler to a heat block) for the detection of *Staphylococcus aureus* enterotoxin A and B genes.

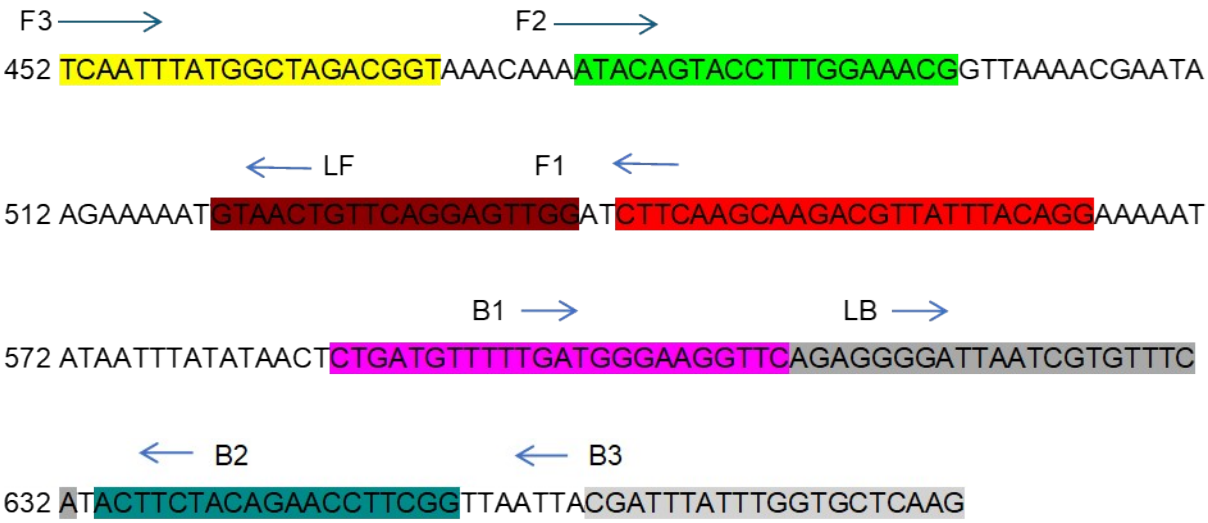


Figure S2. Modified loop-mediated isothermal amplification (LAMP) primer set for detection of *Staphylococcus aureus* enterotoxin A gene, where F3 is the forward primer (yellow highlight), B3 is the backward primer (light grey highlight), LF is the loop forward primer (dark red highlight), LB is the loop backward primer (dark grey highlight), F1 and F2 are the sequences of the inner forward primer (red and green highlights, respectively) and B1 and B2 are the sequences of the inner backward primer (purple and dark green highlights, respectively). The arrows refer to the direction of the primers.

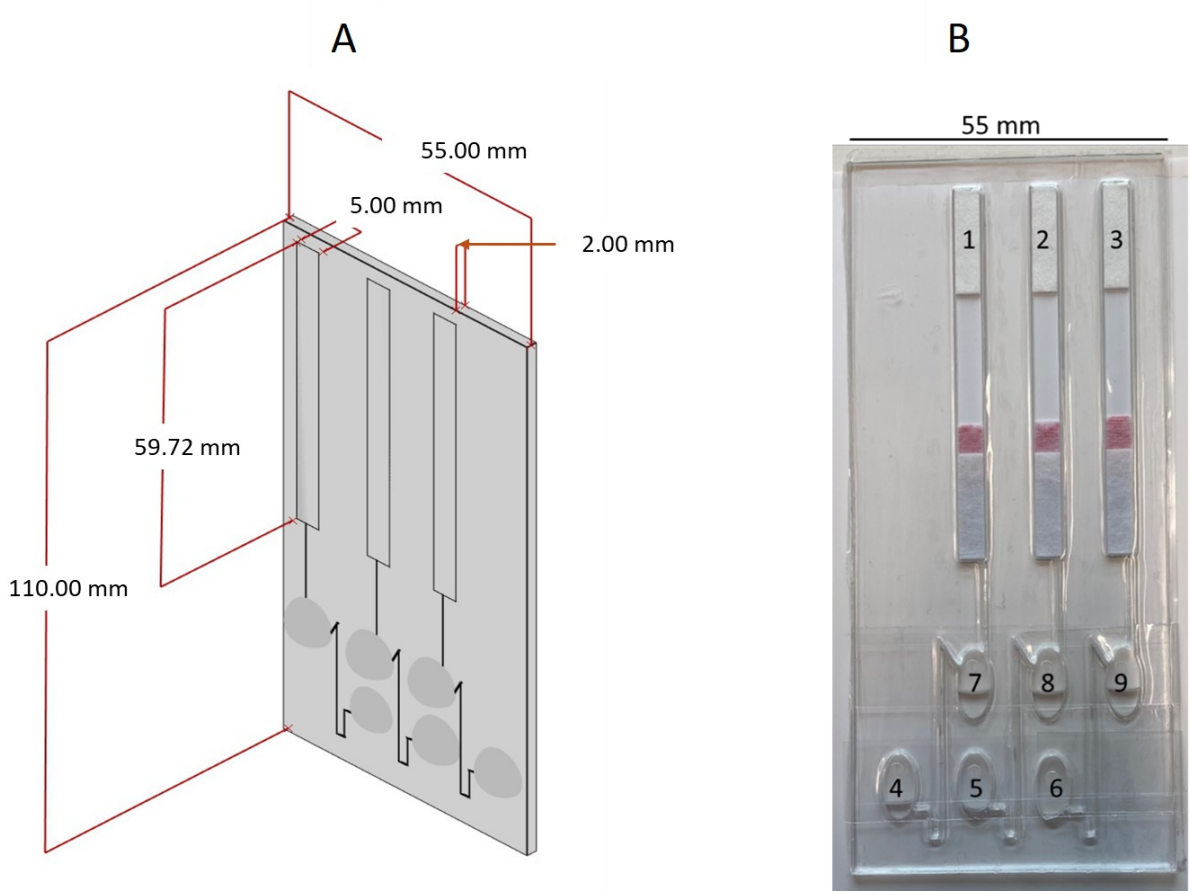


Figure S3. (A) Dimensions of the microfluidic device for detection of *Staphylococcus aureus* enterotoxin genes without DNA extraction. This was drawn using FreeCAD software (V 0.18). (B) Fabricated microfluidic device for testing milk samples. Detection strips 1, 2 and 3 are for positive control, negative control and sample, respectively. Three chambers (finger pumps; 4, 5 and 6) enclose the diluent, while the other three chambers (7, 8 and 9) contain the master mix.


Figure S4. A picture of the developed device inserted in the heat block to run the optimised multiplex loop-mediated isothermal amplification (LAMP) assay for detection of *Staphylococcus aureus* enterotoxin A and B genes in raw ruminants' milk.

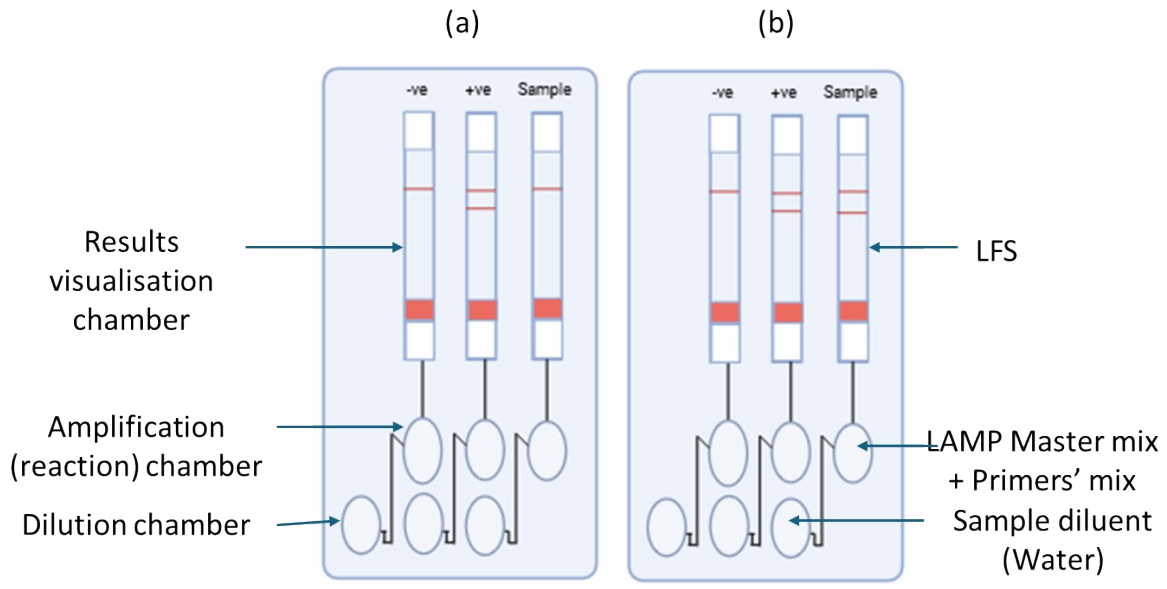


Figure S5. Negative (a) and positive (b) sample results on developed microfluidic device for detection of *Staphylococcus aureus* enterotoxin genes. From right to left, lanes show sample and positive (+ve) and negative (-ve) controls. LFS is lateral flow strip and LAMP is loop-mediated isothermal amplification.

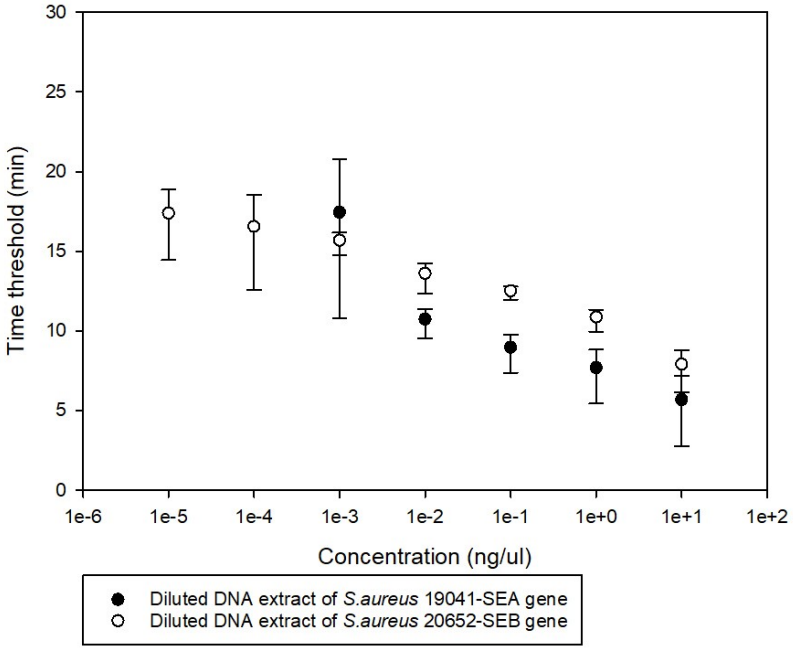


Figure S6. Limit of detection (LOD) of loop-mediated isothermal amplification (LAMP) assay for *Staphylococcus aureus* enterotoxin A and B genes (SEA and SEB) in diluted DNA extract of overnight culture of *Staphylococcus aureus* DSMZ 19041 and 20652 reference strains, respectively. Each concentration was performed in triplicates and lower concentrations of SEA gene were not detected. Error bars are standard deviation.

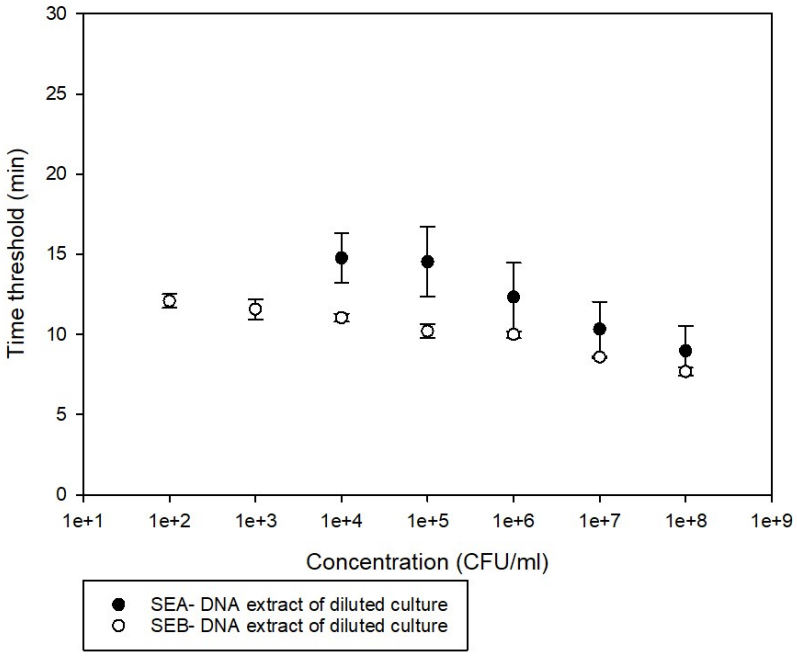


Figure S7. Limit of detection of loop-mediated isothermal amplification (LAMP) assay for *Staphylococcus aureus* enterotoxin A and B genes in DNA extract of each dilution of diluted overnight culture of *Staphylococcus aureus* DSMZ 19041 and 20652 reference strains, respectively. Each concentration was performed in triplicate and lower concentrations of SEA gene were not detected. Error bars are standard deviation.

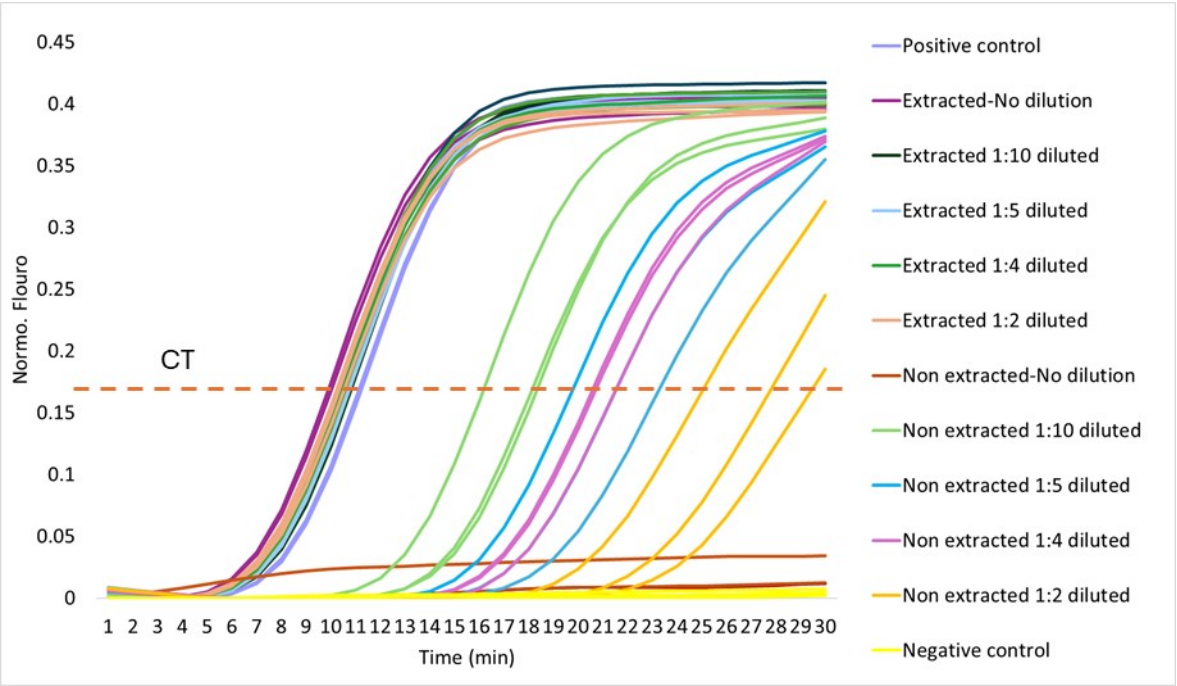


Figure S8. Loop-mediated isothermal amplification (LAMP) assay amplification curves of the *Staphylococcus aureus* enterotoxin B gene (SEB), using cow milk samples spiked with *S. aureus* DSMZ 19041 reference strain then diluted to different dilution factors (shown in legend) with and without DNA-extraction. Each dilution was run in triplicate. CT = cycle threshold value.

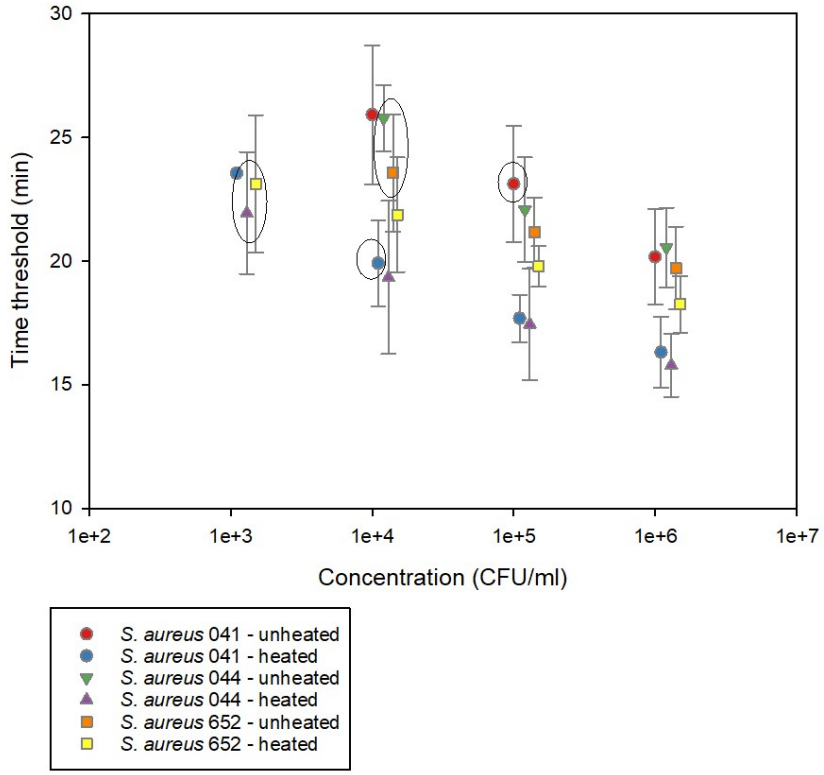


Figure S9. Limit of detection (LOD) of loop-mediated isothermal amplification (LAMP) assay for *Staphylococcus aureus* enterotoxin B (SEB) gene in 1:10 diluted (10 µl spiked milk : 90 µl nuclease-free PCR-grade water) cow milk samples spiked with cells collected from ten-fold serially diluted overnight culture of *S. aureus* DSMZ 19041, 19044 and 20652 reference strains with and without heating. Assays were performed on three independent samples with three technical replicates at each concentration. LOD values are circled for ease of visualisation and data were artificially shifted (jittered) along the x-axis to facilitate visualization. Error bars are standard error. For the $10^3$ CFU/ml concentration of heated spiked and diluted milk samples, error bars are not shown as it was only detected only five times out of nine.

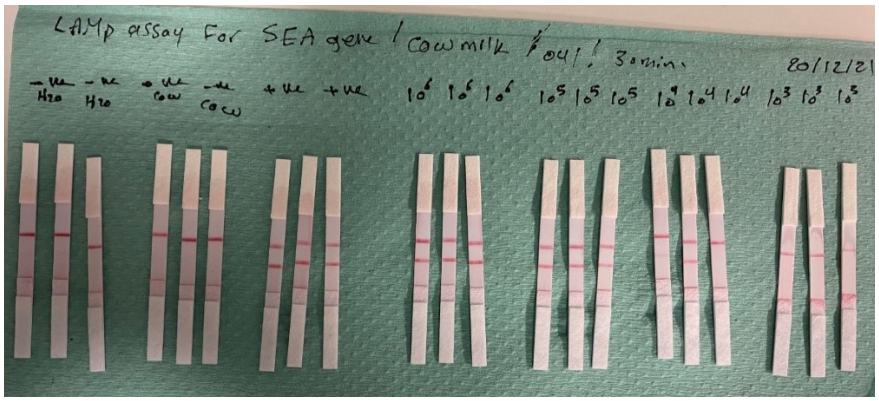


Figure S10. Limit of detection of loop-mediated isothermal amplification (LAMP) assay for detection of *Staphylococcus aureus* enterotoxin A (SEA) gene using spiked 1:10 diluted cow milk samples with different concentrations of *S. aureus* DSMZ 19041 reference strain.

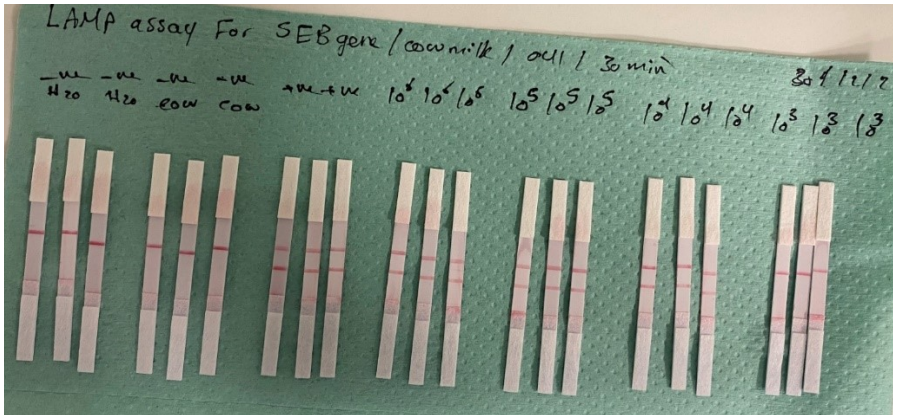


Figure S11. Limit of detection of loop-mediated isothermal amplification (LAMP) assay for detection of *Staphylococcus aureus* enterotoxin B (SEB) gene using spiked 1:10 diluted cow milk samples with different concentrations of *S. aureus* DSMZ 19041 reference strain.

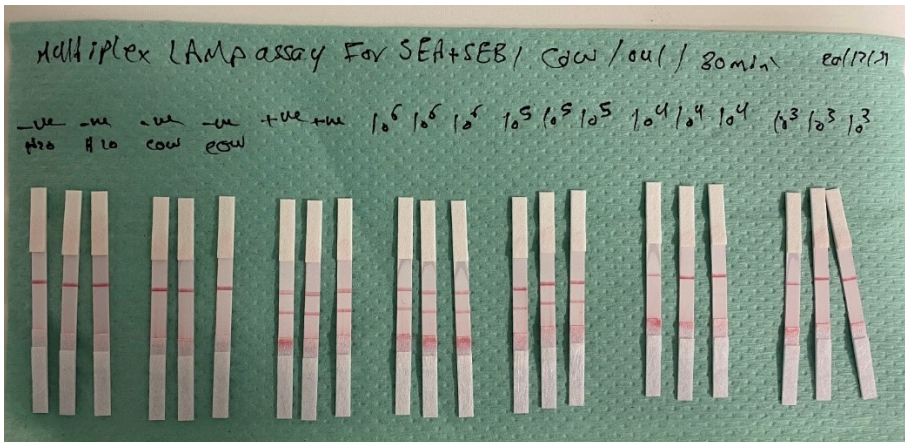


Figure S12. Limit of detection of multiplex loop-mediated isothermal amplification (LAMP) assay for detection of *Staphylococcus aureus* enterotoxin A and B (SEA and SEB) genes using spiked

1:!0 diluted cow milk samples with different concentrations of the *S. aureus* DSMZ 19041 reference strain.

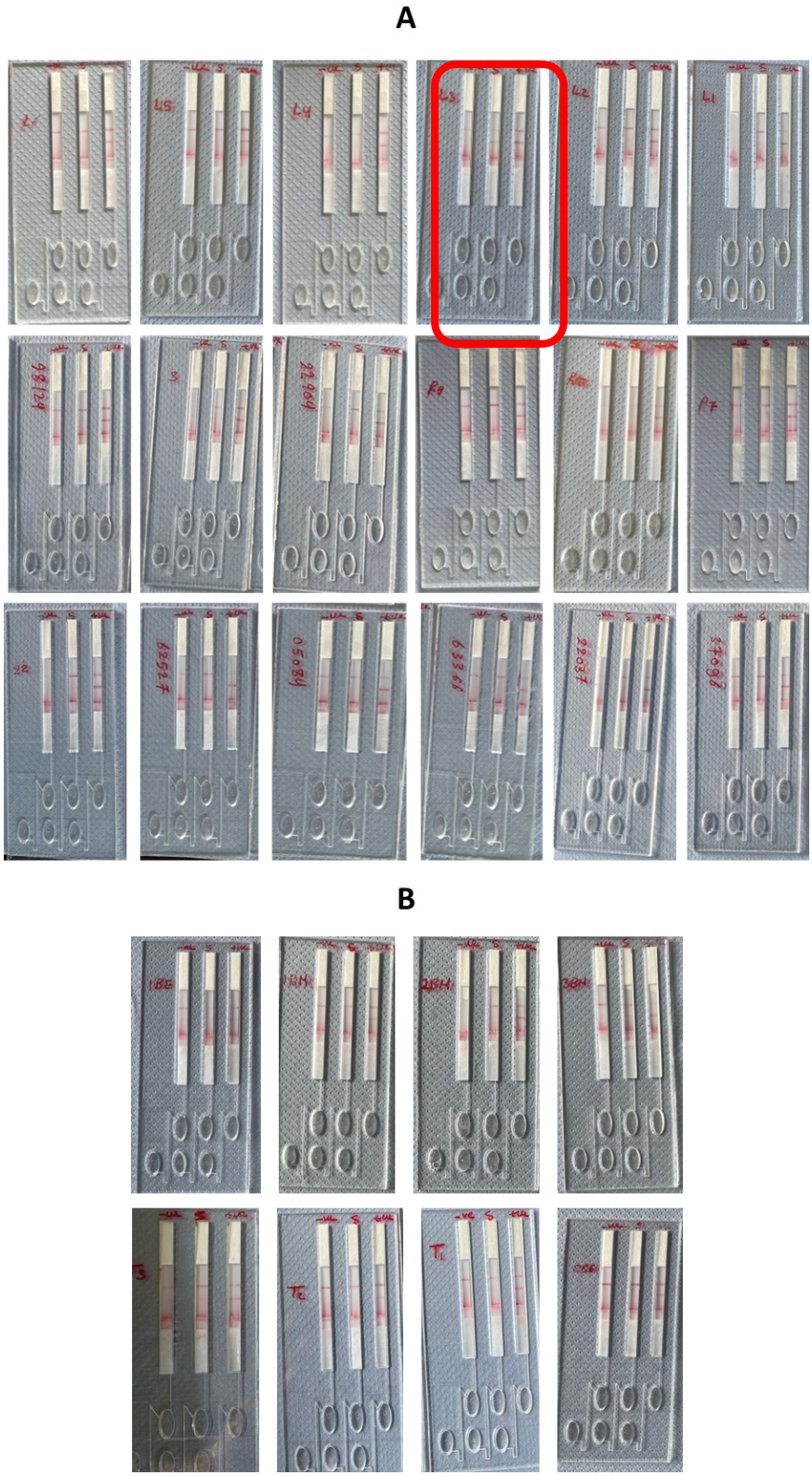


Figure S13. Field testing of (A) individual and (B) bulk tank milk samples for the detection of *Staphylococcus aureus enterotoxins* A and B genes using the developed microfluidic device. A

single result, circled in red, was interpreted as positive whereas the remaining samples were considered negative.

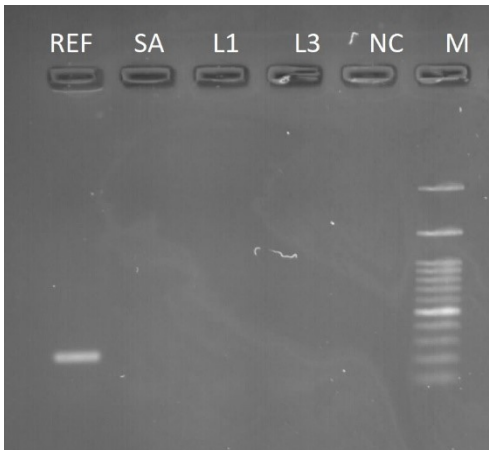


Figure S14. Multiplex PCR assay for detection of enterotoxin A and B genes in the suspected staphylococci isolates of the milk samples tested in the field. REF = Reference bacterial strain carrying B and D enterotoxin genes; SA = *S. aureus* ATCC 25923 (non-enterotoxigenic *S. aureus* – does not carry any enterotoxin genes); L1 = one of the two samples with suspected colonies; L3 = the other sample with suspected colonies and positive with the LAMP assay during field testing; and NC = Negative control (including DNA/RNA free distilled water). M = 100 bp molecular weight ladder.

**References:**

Goto, M., H. Hayashidani, K. Takatori, and Y. Hara-Kudo. 2007. Rapid detection of enterotoxigenic *Staphylococcus aureus* harbouring genes for four classical enterotoxins, SEA, SEB, SEC and SED, by loop-mediated isothermal amplification assay. *Letters in applied microbiology* 45 (1):100-107.

Mehrotra, M., G. Wang, and W. M. Johnson. 2000. Multiplex PCR for detection of genes forStaphylococcus aureus enterotoxins, exfoliative toxins, toxic shock syndrome toxin 1, and methicillin resistance. *Journal of Clinical Microbiology* 38 (3):1032-1035.

Yin, H., T. Fang, and H. Wen. 2016. Combined multiplex loop-mediated isothermal amplification with lateral flow assay to detect sea and seb genes of enterotoxic Staphylococcus aureus. *Letters in applied microbiology* 63 (1):16-24.