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Sample-to-answer microfluidic device towards the point-of-need detection of *Staphylococcus aureus* enterotoxin genes in ruminant milk†

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Milk is commonly screened both for indicators of animal disease and health, but also for foodborne hazards. Included in these analyses is the detection of *Staphylococcus aureus*, that can produce an enterotoxin, causing staphylococcal food poisoning (SFP), which often leads to sudden onset of significant gastrointestinal symptoms in humans. Epidemiological data on SFP are limited, particularly in low- and middle-income countries. Many conventional assays for the detection of staphylococcal enterotoxins rely on the detection of the genes coding for them, either directly in food samples or after bacterial culture. Currently, many of the nucleic acid-based methods used require specific expertise and equipment, whilst bacterial culture takes 24–48 hours; both are contributory factors that limit efforts either during food safety emergencies or routine screening. Here we present the development of a "sample-to-answer" isothermal nucleic acid loop-mediated amplification (LAMP) assay in a microfluidic device for the detection of *Staphylococcus aureus* enterotoxin genes in ruminant milk. A multiplex LAMP assay targeting two of the most prevalent *S. aureus* enterotoxin-encoding genes (A and B) was integrated into a microfluidic device combining simple 1:10 dilution for sample preparation and a lateral flow assay for easy readout. We achieved a limit of detection of 10^4 colony forming units per ml in spiked cow and goat milk samples, an order of magnitude more sensitive than the European recommendation for the maximum allowable presence of coagulase-positive staphylococci in raw milk. The assay showed no cross-reactivity in detecting other tested non-enterotoxigenic *S. aureus* strains or associated foodborne pathogens. The test integrated the simplicity of use of microfluidic devices with the sensitivity, specificity and rapidity of a nucleic acid-based assay, and a simple lateral flow readout to provide an appropriate device to ensure the safety of milk for human consumption. To illustrate its potential for point-of-need practical applications, the test was performed in agricultural settings in rural Turkey in a limited feasibility exercise.

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1. Introduction

Staphylococcal food poisoning (SFP) occurs following the consumption of food containing sufficient amounts of one (or more) preformed staphylococcal enterotoxins (SEs).[1,2]

Symptoms can arise from as little as 0.5 ng of toxin per mL of milk.[3] These enterotoxins are encoded by SE genes, which form part of the staphylococcal virulence gene repertoire,[1,4] and are serologically classified into classical antigenic SEs (A-J) and the newly described group of SEs (K-U).[5–7] Around 95% of reported SFP cases are caused by the classical staphylococcal enterotoxins,[8,9] with SEA and SEB most commonly causing gastrointestinal symptoms.[10] SFP symptoms comprise nausea, vomiting and abdominal cramping, with or without diarrhea, often with rapid onset (2–8 hours after exposure).[11,12] It is usually a self-limiting disease, with requirement of hospitalization in some cases, particularly in children, the elderly and in people with co-morbidities.[4,13]

S. aureus is an opportunistic pathogen that can be carried by or infect various hosts, including humans and domesticated ruminants (cows, buffalo, sheep and goats).[14] It

is commonly reported as a cause of clinical and sub-clinical mastitis (*i.e.*, inflammation of the udder) in dairy animals.[15–17] Humans are considered as the most common source of *S. aureus* contamination involved in SFP, however, animals with *S. aureus* carriage or active infections are also recognized as an important source of contamination with enterotoxigenic *S. aureus*, for instance in raw animal products (*i.e.*, raw milk, milk products, and raw meat products).[18,19]

Staphylococcal growth and toxin production is commonly promoted by inadequate storage, cooking or handling, as well as cooling of food contaminated with enterotoxigenic *S. aureus* of human or animal origin.[20] One of the common routes of SFP is consumption of milk and milk products, especially fresh dairy products.[21] It is also noteworthy that although pasteurisation temperatures are sufficient to destroy the pathogen, such treatments do not inactivate the enterotoxins,[22] which require prolonged boiling or autoclaving to gradually decrease their potency (Ortega *et al.* 2010). In the case of ultra heat-treated milk (UHT milk), the heating temperature should, theoretically, be sufficient, although the overall efficacy is affected by the toxin's initial concentration.[23,24]

Specific incidence data of SFP outbreaks are limited, with available reports from only some global subregions.[25,26] The limitation of data availability is most problematic in low- and middle-income countries, most probably as many affected people do not seek medical attention. In addition to the lack of routine surveillance tools, the limited availability of clinical specimens and the complicated laboratory confirmation protocols all contribute to under-reporting.[27] The number of SFP outbreaks reported in the European Union increased from 25 outbreaks between 2007 and 2011[28] to 117 outbreaks in 2019 and 2020[29,30] whilst in the United States, 42 outbreaks were recorded between 1993 and 1997[31] and 75 outbreaks between 2009 and 2015.[32]

According to data reported in our recent study, there has been a gradual change in the methods employed for the detection of *S. aureus* enterotoxins, where nucleic acid amplification tests (NAAT, identifying the genes) have been replacing the detection of the toxins themselves,[26] opening up the potential for the development of new the point-of-need technologies.[33,34] Within the context of the dairy value chain, detecting toxins or a risk of contamination either at the milking stage next to the animal, or within the dairy storage tank on the farm, would allow an early intervention to prevent the milk from entering the food chain. Such detection at the point-of-need is important for food safety.

Several assays have been developed for the detection of *S. aureus* enterotoxin genes, either directly from food samples using molecular tools or after an enrichment and/or culture step to increase bacterial concentrations.[35] However, these assays are time-consuming,[36] labor-intensive,[35] and often require significant expertise and sophisticated equipment,[37] limiting their application in food safety emergencies, such as SFP outbreaks.

Isothermal methods, such as loop-mediated isothermal amplification (LAMP), eliminate the need for thermal cycling, and can often be performed in a single step in a single tube.[38] Laboratory-based LAMP assays have already been developed for the detection of enterotoxin genes of *S. aureus*, either as a single or multiplex assay, with a high analytical sensitivity (limit of detection (LOD) of 10 and 10^2 CFU ml^{-1}, respectively) and specificity (100%).[39,40] However, these assays still require access to a laboratory facility, which delays detection and subsequently the response to potential disease outbreaks.

To overcome the limitations of these laboratory-based assays, microfluidic technologies have emerged as portable, rapid and cost-efficient platforms that could be deployed at the point-of-need and thus provide near real-time responses to emergencies.[41] Such methods have previously been applied to the detection of methicillin-resistant *S. aureus*; however these have still shown barriers for applicability in the field. For example, a study reporting a microfluidic-based PCR assay required pre-treatment of the sample to extract nucleic acids[42] and a microfluidic LAMP-based assay required access to pneumatic and control systems to drive fluids through complex processes,[43] preventing their wider application at the point-of-need.

With the goal of moving towards point-of-need applications, in this work, we have developed a prototype "sample-to-answer" LAMP-based microfluidic point-of-need device for the detection of *S. aureus* enterotoxin genes in ruminant milk (cow and goat). The test combines simple sample preparation steps (including direct dilution) integrated onto a microfluidic device, combined with incubation in a heat block and lateral flow strip detection (Fig. 1).

2. Materials and methods

2.1 *Staphylococcus aureus* reference strains: source and maintenance

Enterotoxigenic *Staphylococcus aureus* reference strains were purchased from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (ESI† Table S1). To evaluate the specificity of the LAMP assay, reference strains of non-enterotoxigenic *S. aureus* (*S. aureus* 171.1), non-aureus staphylococcal strains (*Staphylococcus epidermidis* 49510 and *Staphylococcus intermedius* 63687) and non-staphylococcal species commonly causing food poisoning (*Salmonella enteritidis* 67199 and *Bacillus cereus* 48306) were sourced from Veterinary Laboratory Quality Assurance scheme provided by the Veterinary Laboratories Agency and identified by Scottish methicillin resistant *S. aureus* reference laboratory.

Reference strains were grown on brain heart infusion (BHI) agar (Sigma Aldrich) at 37 °C overnight, then inoculated into BHI broth (Sigma Aldrich) (one colony for each tube) and incubated at 37 °C overnight. After adjusting concentrations (using optical density at 600 nm) to ~1 OD$_{600}$
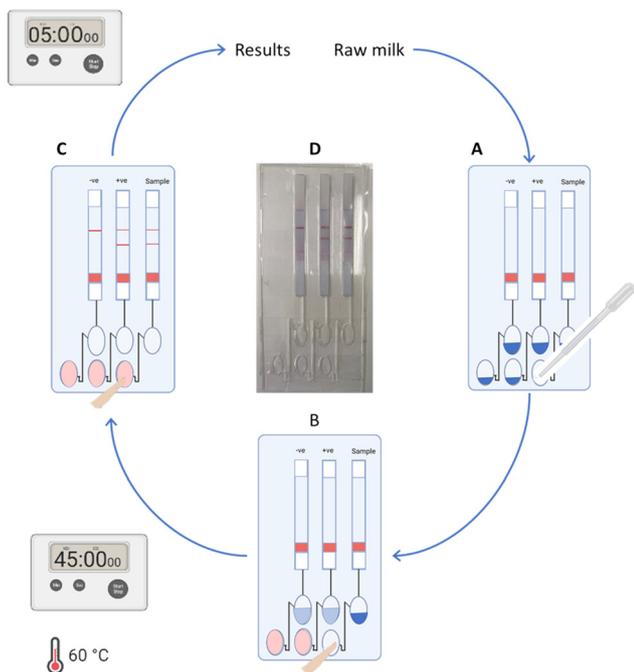
Fig. 1 Schematic diagram showing the process and structure of the prototype point-of-need test. (A) After adding the milk to the diluent chamber containing PCR-grade nuclease-free water, effecting a finger pump on the dilution chamber results in the sample being diluted and mixed with LAMP reagents present in the amplification chamber. (B) The amplification reaction occurs at 60 °C for 45 minutes. (C) The amplicons are labelled with biotin and fluorescein isothiocyanate (FITC) through the primers, leading to binding to the test line and streptavidin-coated beads for visualisation on a lateral flow strip. (D) The process was integrated into a simple microfluidic format, including dilution, amplification and read out, using finger pumps to move liquids through the chip. The lower three ovals with dark blue color represent the dilution chambers with the diluent, and those with pink color represent the dilution chambers after finger pumping the diluted sample to the amplification chamber. The upper three ovals with dark blue color represent amplification chambers with amplification reagents (master mix and LAMP primer mix) and with light blue color represent the mixture of diluted sample with amplification reagents. From left to right, the first strip is a negative control, showing only the control line, while the two strips for positive control and sample show both the control line and the test line, illustrating a positive test, *i.e.* indicating amplification of SEA and/or SEB (C and D).

using a spectrophotometer (Eppendorf Bio-photometer V1.35), they were ten-fold serially-diluted in sterile BHI broth, and colony counts were determined by conventional plate counting (using three 10 µl drops per dilution, and counting those drops with 5–50 colonies). Stocks from overnight BHI broth cultures of each reference strain were maintained in 15% glycerol from BHI broth cultures and stored at −80 °C.

2.2 Evaluation of specificity and sensitivity of LAMP assay for detection of *Staphylococcus aureus* enterotoxin A and B genes

The different steps taken for the evaluation, optimisation and transfer of LAMP assays for detection of *S. aureus*

enterotoxin A and B genes (SEA and SEB) are summarized as a diagram in ESI† Fig. S1.

The SEA gene LAMP primer set was based on published primers,[40] with modifications to increase assay specificity. The new loop forward primer was designed using Primer Explorer V5web server (Eiken Chemical Co. LTD, Tokyo, Japan; **http://primerexplorer.jp/e**),[44] based on the SEA sequence obtained from GenBank accession number M18970, and manual modification of the position of the F1 primer (ESI† Table S2 and Fig. S2). The SEB LAMP primer set was used as described[40] (ESI† Table S2). The primers were BLASTed against the NCBI nucleotide collection and IDT OligoAnalyzer™ Tool was used to check for formation of hairpin, self-, and hetero-dimers. Artificial oligonucleotide dsDNA sequences (Integrated DNA technologies (IDT)) for each gene were designed manually for use as positive controls, mapping the target region of each primer set for both genes (ESI† Table S3).

Cross reactivity of SEA and SEB LAMP primers was evaluated against the artificial oligonucleotide dsDNA sequences of the other gene, using high (9.3×10^{10} and 1.78×10^{11} copies per µl) and low (9.3 and 1.78 copies per µl) concentrations (to ensure the accuracy of the assay, even when low concentrations of cross-reactants might be present) of SEA and SEB artificial dsDNA oligonucleotide sequences, respectively. Specificity of the LAMP assay for the detection of SEA and SEB genes was evaluated using DNA extracts of overnight cultures of reference strains from non-enterotoxigenic *Staphylococcus aureus*, non-aureus staphylococcal strains and other non-staphylococcal species commonly causing food poisoning (described in 3.1). The analytical sensitivity of the LAMP assay for the detection of SEA and SEB genes (LOD) was determined in two ways: (i) using DNA extracts of overnight cultures of the three *S. aureus* reference strains, which were subsequently diluted, and (ii) by preparing ten-fold serial dilutions of overnight cultures of the three *S. aureus* reference strains (*S. aureus* DSMZ 19041, *S. aureus* DSMZ 19044 and *S. aureus* DSMZ 20652), from which DNA was subsequently extracted. The LOD was identified as the lowest concentration detected for all the replicates (three biological and three technical replicates).

Genomic DNA for testing was prepared as follows: from each overnight BHI broth culture of each reference strain, cells were harvested from 1 ml by centrifugation at $9600 \times g$ (10 000 rpm) for 10 minutes and DNA was extracted using a MagaZorb® DNA Mini-Prep Kit (Promega) following the manufacturer's protocol. The quantity of DNA in the extract of each culture was determined using a Nanodrop 1000 Microvolume spectrophotometer (Thermofisher), and the quality of the DNA was confirmed by ensuring that it had an A260/A280 ratio >1.8.

2.3 Sample preparation optimisation

To evaluate the effect of dilution, instead of DNA extraction, as a simple sample preparation step, homogenized

pasteurized whole cow and goat milk samples (purchased from commercial food outlets, on different occasions as biological replicates) were spiked with cells collected from overnight culture of *S. aureus* DSMZ 19041 reference strain (1 ml of the overnight culture was centrifuged at 1000 rpm for 10 minutes, the supernatant was discarded and the collected cells were resuspended in PBS and used to spike milk samples), then diluted (1 : 2, 1 : 4, 1 : 5 and 1 : 10); each dilution was tested with three biological and three technical replicates. Milk samples that had been spiked and diluted were used with and without DNA extraction to run LAMP assays.

In addition to diluting milk samples spiked with a single concentration of *S. aureus*, we also considered milk samples spiked with different concentrations of *S. aureus*. Homogenized pasteurized whole cow and goat milk samples were spiked with bacterial cells collected from ten-fold serially diluted overnight cultures of each *S. aureus* DSMZ 19041, 19044 and 20652 reference strains. Each overnight culture was serially diluted by adding 1 ml of the culture to 9 ml of sterile broth media. Bacterial cells from each dilution were collected by centrifugation at 1000 rpm for 10 minutes, the supernatant was discarded, and the cells were resuspended in PBS. These resuspended cells were then used to spike the milk samples. Following spiking, each milk sample with the corresponding bacterial concentration was further diluted 1 : 10 and directly used in the LAMP assay without any DNA extraction.

In a similar process as that described above for dilution, the following two approaches were assessed for their impact on analytical sensitivity: i) heating pre-treatment was added as another simple physical sample preparation step to explore the potential benefits for bacterial lysis, incubating at 95 °C for 10 minutes before running the assay; and ii) increasing the assay time (60 °C isothermal amplification) to 60 minutes instead of 30 minutes.

2.4 LAMP assay optimisation

The LAMP reaction for each gene (SEA and SEB) was carried out in a 20 μl reaction mixture containing 0.8 μl (10 pmol μl^{-1}) of each inner primer (FIP and BIP), 0.2 μl of each outer primer (F3 primer and B3 primer), 1 μl (10 pmol μl^{-1}) of each loop primer (LF and LB), 1 μl nuclease-free PCR-grade water (Invitrogen) and 15 μl of GspSSD2.0 Isothermal Master Mix (ISO_004) (OptiGene). The duplex LAMP assay was also carried out in a 20 μl reaction mixture containing half of the primers' volume from individual reactions. A volume of 5 μl of the sample under investigation (1 : 10 diluted spiked milk samples, or relevant controls) was added to each reaction (total volume 25 μl). The reaction mixture was incubated in a heat block (in tubes) for 30, 45 and 60 minutes, or a Rotor-Gene Q L2-0694-00 thermocycler (Qiagen), with an initial cycle of 60 °C for 20 seconds, followed by 30 cycles of 60 °C for 1 minute then termination at 95 °C for 10 minutes. Negative control reactions using nuclease-free PCR-grade

water, PBS (used for reconstitution of collected cells from overnight cultures of reference strains), media (BHI broth) and cow/goat milk, as well as positive controls using artificial dsDNA oligonucleotide sequences, were prepared with each run to screen for the contamination of the reagents, and to confirm that the assay was working properly.

2.5 Statistical analysis

Statistical analysis was performed using SIGMAPLOT (v13.0), using a two-way ANOVA to compare the difference in amplification time associated with different factors including dilution, heating, assay time, enterotoxigenic *S. aureus* strains, single-plex and duplex assays, and milk sources (cow and goat). *P*-Value < 0.05 was considered significant based on the studentized range distribution (*q* value).

2.6 Design, fabrication and manipulation of microfluidic device

Plastic microfluidic devices were designed using Inkscape (v 0.92.4) and FreeCAD software as previously described,[34,45] and fabricated using CNC machining by Epigem Ltd, sealed with adhesive acetate film on both sides (Fig. 1D). The dimensions are provided in the ESI† (Fig. S3A). The device comprised six finger pump chambers, three for sample preparation (containing sample diluent, *i.e.*, nuclease free water) and three for amplification (containing LAMP master mix and primer mix), as well as three chambers containing lateral flow strips (LFS) for visualisation of results (ESI,† Fig. S3B).

Eighteen μl of sample diluent (nuclease free water) and 30 μl master mix (containing primers) were loaded separately and the chambers were sealed. The volumes were adjusted to meet the requirements for the lateral flow strip wetting (without the need for an extra step for adding running buffer), which needs ∼40 μl of liquid. This represents the final state of a manufactured device, as would be presented to the user before testing. For the test, 2 μl of sample was added into the diluent chambers, which were then sealed with adhesive acetate film. The finger pumps on the sample diluent chambers were actuated to both mix the sample and move all components into the LAMP reaction mix. The devices were inserted in the heat block to run the LAMP assay at 60 °C (ESI,† Fig. S4). The LAMP reaction products were then transferred to the lateral flow strips using the same finger pumps again, then the assay proceeded under capillary flow for 2 minutes before results were analysed by eye. The diagnostic device included a negative control strip (with just PCR grade nuclease free water as the sample, pre-loaded in the device) and a positive control consisting of SEA and SEB targets (1 ng μl^{-1}), amplified from artificial dsDNA oligonucleotide sequences. This quality control step allows false positives arising from contamination of reagents to be ruled out, and false negatives related to the assay reagents, respectively (ESI,† Fig. S5).

2.7 Field testing

For preliminary evaluation of robustness and user friendliness under field conditions using raw milk samples, we collected 26 samples over two days in one of the production dairy farms of Ataturk University, Turkey with total capacity of 143 cows: 18 samples from individual cows (composite; *i.e.*, the four udder quarters were sanitized with iodine, then the milk was collected from each quarter and combined) and eight bulk tank samples. Samples were tested on the farm using the microfluidic device for the detection of *S. aureus* enterotoxin A and B genes as per 2.6 above. For volume accuracy, a micropipette was used but could be replaced by droppers[46,47] and Pasteur pipettes[48] in future.

As a comparator assay, performed blind to the field results by a different person, all 26 milk samples were streaked on sheep blood agar containing 7% sheep blood, MacConkey agar, and Mannitol Salt agar (all Oxoid) and incubated at 37 °C for 24 hours. Growing colonies of each morphotype were tested by Gram stain. Isolated Gram-positive cocci were identified as members of the genus *Staphylococcus* using routine biochemical tests (*e.g.*, catalase, coagulase, DNase, haemolysis on blood agar, urease, mannitol fermentation on mannitol salt agar). They were also analysed for susceptibility to bacitracin and polymyxin B antibiotics and carbohydrate fermentation tests.[49] For SEA and SEB gene confirmation, DNA was extracted from biochemically-confirmed staphylococcal isolates using the boiling method. Briefly, two overnight colonies were transferred to a 0.2 ml PCR tube and dissolved in 40 μL single-cell lysis buffer (including Tris–HCl, disodium EDTA, and TE Buffer). The tube was heated to 80 °C for 10 minutes and cooled down to 55 °C for 10 minutes in a thermocycler (Kyratec SC300). The resulting suspension was diluted with PCR-grade nuclease-free water and centrifuged at 4500 g for 30 s to eliminate debris. The supernatant (containing the DNA) was used to run a multiplex PCR assay. The primers used for the detection of SEA and SEB genes were as described[39,50] (ESI† Table S4). The reaction mixture used was the same as the multiplex LAMP mixture mentioned before (section 3.4). A reaction mixture containing no added DNA and another with the DNA extract of non-enterotoxigenic *S. aureus* reference strain (*S. aureus* ATCC 25923) were used as a PCR negative controls. A reaction mixture containing the DNA extract of enterotoxigenic *S. aureus* reference strain carrying enterotoxin B gene was used as a positive control. The thermal running conditions were: 95 °C for 5 minutes; 30 cycles of 95 °C for 30 seconds, 58 °C for 30 seconds and 72 °C for 1 minute; 5 min at 72 °C. PCR products of each strain were analysed by gel electrophoresis, with expected band size of 102 and 164 bp for SEA and SEB genes, respectively.

3. Results and discussion

3.1 Evaluation of specificity and sensitivity of a LAMP assay to detect *Staphylococcus aureus* enterotoxin A and B genes

We first assessed the cross reactivity and specificity of the LAMP primer sets, and noted that there was no amplification
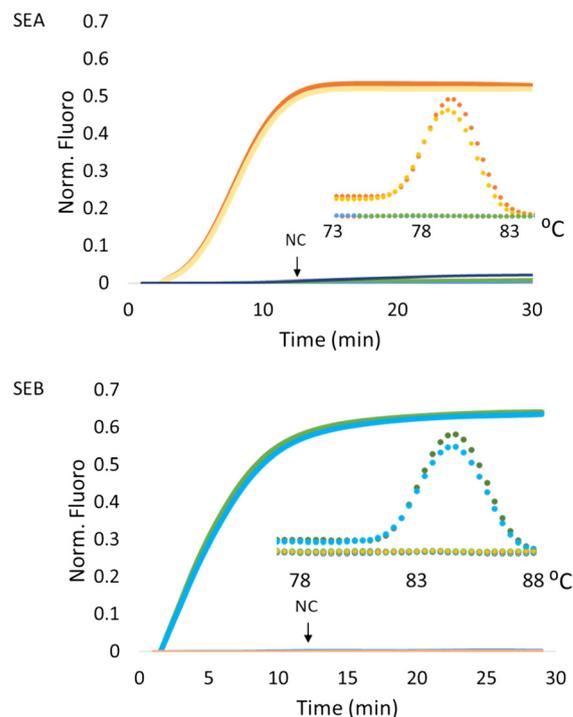
Fig. 2 Loop-mediated isothermal assay (LAMP) amplification (solid lines) and melt curves (dotted lines; insets) for *Staphylococcus aureus* enterotoxin A (SEA) and B (SEB) genes. Amplification curves were only observed for positive controls using the artificial oligonucleotide dsDNA sequence of each gene with its LAMP primers set. Specificity of the assay was confirmed, with no amplification observed when testing i) a non-enterotoxigenic *Staphylococcus aureus* strain; ii) non-aureus staphylococcal strains (*Staphylococcus epidermidis* and *Staphylococcus intermedius*), iii) other foodborne pathogens (*Salmonella enteritidis* and *Bacillus cereus*), and iv) when SEA or SEB primer sets were used to amplify the artificial oligonucleotide dsDNA sequence of the other gene (all in negative control test). Each sample was run in duplicate. The two orange (light and dark) curves represent the two replicates of positive control for SEA gene, while dark green and light blue curves represent the two replicates of positive control for SEB gene.

with either SEA or SEB primer sets when testing high and low concentrations of the artificial dsDNA oligonucleotides sequences of the other gene, nor with any of the other non-enterotoxigenic reference strains evaluated (Fig. 2).

The LOD of the LAMP assays for the detection of SEA and SEB genes from bacterial cultures was established through serial dilutions of DNA extracts, reaching 10^{-3} ng μl^{-1} in diluted DNA extracts and 10^4 CFU ml^{-1} in DNA extracts from diluted cultures for the SEA gene, whilst it was 10^{-5} ng μl^{-1} and 10^2 CFU ml^{-1} for the SEB gene (ESI† Fig. S6 and S7).

The LOD for the SEB gene when using extracted DNA (10^2 CFU ml^{-1}) was comparable with those available from literature.[39,40] Whilst the LOD for the SEA gene was higher than that previously reported, we speculate that this may be due to modifications made to the associated primer set (which was done to improve specificity), primer concentration and/or the fact that we used a different DNA extraction kit than the one described in the previous

publication(s).[39,40] Our modified assay also could detect the SEA gene in a culture with bacterial concentration lower than that needed for enterotoxins to be formed (10^5 CFU ml^{-1}), and thus stipulated as the required LOD by European regulation.[51] The LOD was calculated as the lowest concentration consistently detected for all the replicates. In this work, we use the LOD as a performance parameter to study the different implementations of the assay and its optimisation. The LOD for detection of the SEA and SEB genes in milk samples was determined as part and parcel of the optimisation of sample preparation.

3.2 Sample preparation optimisation

Milk sample dilution in water resulted in faster amplifications as dilution was increased. For 1:2 (0.5) diluted samples, amplification was detected in all three technical replicates among one of three biological replicates after 27–28 minutes of amplification, whereas 1:10 diluted samples (0.1) amplified within 17–18 minutes (Fig. 3). By contrast, amplification was not detected when using undiluted milk samples for LAMP. Extracted DNA from spiked milk samples could be detected at 7–8 minutes. Amplification curves of the SEB gene, using spiked diluted cow milk samples with and without DNA extraction, are shown in the ESI† Fig. S8.

A key challenge in using milk samples for molecular assays is the inhibitory nature of milk components,[52] such that sample preparation steps have always been required before molecular analysis of milk samples.[53] These preparation steps often include chemical treatment, *e.g.*, using DNA extraction kits and lysis buffers[54] and are time

consuming, costly and require trained personnel, which is not suitable for field application.[55] Many approaches have been developed to simplify the sample preparation process,[56] including, for example, physical preparation such as centrifugation,[57] heating,[58] filtration,[59] and dilution,[60] or often several such steps in combination.

In our approach, we used only a single dilution, one of the simplest modes of physical preparation, which has been shown to facilitate cell lysis through osmotic shock[61] when combined with increased LAMP temperature (lysis and heating shock) and could also provide efficient dilution of the inhibitory components in milk samples, as also implemented in other assays.[60,62,63] Our results showed that, in general, the dilution of spiked milk samples resulted in significant improvements to the analytical sensitivity of the assay and faster amplification times as dilution was increased. Our results thus indicate that dilution can be used as a simple field-applicable sample preparation step for milk, taking inspiration from other samples (*e.g.* blood[64,65]).

Heating decreased amplification time (*i.e.* time to reach a visible amplification signal) in 1:10 diluted spiked milk samples from both cows and goats (Fig. 4A–C; ESI† Table S5). Heating had no impact on the analytical sensitivity of detection of the SEA gene in cow milk samples spiked with *S. aureus* DSMZ 19041 (LOD was 10^4 CFU ml^{-1} with or without additional heat treatment), while it led to a one log reduction, *i.e.*, an improvement, in the LOD for spiked goat milk samples (10^4 CFU ml^{-1} without heating *vs.* 10^3 CFU ml^{-1} using heating at 95 °C) (Fig. 4A). There was no amplification in any of the replicates or the experiments with cow or goat milk samples spiked with 10^2 and 10^1 CFU ml^{-1}. LOD of multiplex LAMP assay for SEA and SEB genes in 1:10 diluted cow and goat milk samples spiked with *S. aureus* 19041 was 10^4 and 10^5 CFU ml^{-1} with and without heating, respectively (Fig. 4B). Following demonstration that it was possible to detect DNA in milk spiked with bacterial cells following dilution and heat incubation at 60 °C for amplification, we explored if pre-heating at 95 °C for 10 minutes could enhance bacterial cell lysis and thus decrease LOD. Heating has been previously reported to be a simple, affordable, rapid and effective method for DNA extraction from bacteria.[66,33]

Combining sample dilution and heat treatment (*i.e.* lysis at 95 °C for 10 minutes), the LOD in spiked 1:10 diluted cow milk samples with *S. aureus* DSMZ 19041 reference strain with and without heating was 10^4 and 10^5 CFU ml^{-1}, respectively (ESI† Fig. S9). The LOD in 1:10 diluted cow milk spiked with *S. aureus* DSMZ 19044 and 20652 reference strains were 10^3 and 10^4 CFU ml^{-1}, respectively. LOD of SEB gene in 1:10 diluted goat milk samples spiked with each reference strain with and without heating was 10^3 and 10^4 CFU ml^{-1}, respectively (Fig. 4C).

The LOD of LAMP assays for SEA and SEB genes in 1:10 diluted goat milk samples spiked with the cells collected from ten-fold serially diluted overnight culture of *S. aureus* DSMZ 19041 reference strain and incubated at 60 °C for 60
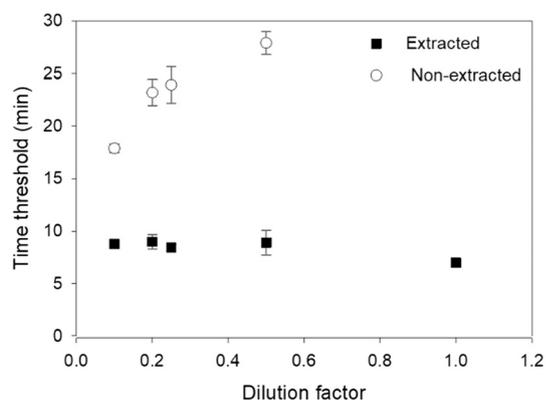
Fig. 3 Evaluation of sample dilution as a preparation step for the detection of *Staphylococcus aureus* enterotoxin B (SEB) gene in spiked cow milk samples using loop-mediated isothermal amplification (LAMP) assay. Black squares: DNA was extracted from cow milk samples spiked with cells collected from overnight culture of *Staphylococcus aureus* DSMZ 19041 reference strain. The samples were then diluted in water (from no dilution to factor 1:10 (0.1)) and subjected to LAMP after (black squares) or without (open circles) DNA extraction. Each dilution was repeated three times (three biological replicates), each with three technical replicates. Error bar represents standard error of the mean.
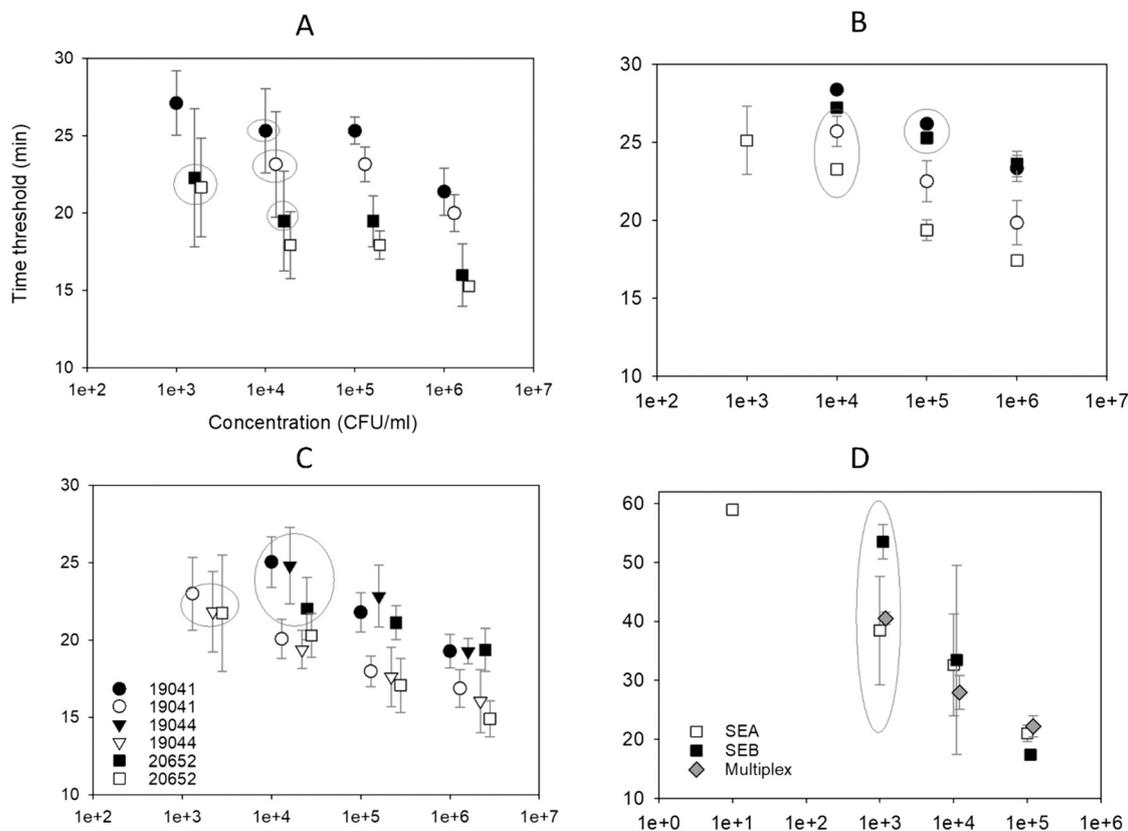
Fig. 4 Limit of detection (LOD) for *Staphylococcus aureus* enterotoxin (SE) genes using loop-mediated isothermal amplification (LAMP). All tests were performed using spiked milk samples diluted 1:10 with molecular grade water. Values for LOD are circled. (A) Amplification of SEA gene; (B) multiplex assay for SEA and SEB genes; and (C) amplification of SEB gene, comparing the effect of no heat treatment (closed symbols) *vs.* heating (open symbols) at 95 °C for 10 minutes prior to LAMP amplification at 60 °C for 30 minutes. For (A) and (B) both cow milk (discs) and goat milk (squares) samples were spiked with *S. aureus* DSMZ 19041 ten-fold serially diluted. For (C) goat milk samples were spiked with *S. aureus* DSMZ 19041, 19044 and 20652 reference strains ten-fold serially diluted (results for cow milk are shown in ESI† Fig. S9). (D) LAMP and multiplex LAMP assays for SEA and SEB genes in goat milk samples spiked with *S. aureus* DSMZ 19041 reference strain incubated at 60 °C for 60 minutes without 95 °C heat treatment. Assays were performed on three independent samples (three biological replicates) with three technical replicates at each concentration. Error bars are standard error. Data were artificially shifted (jittered) along the *x*-axis for ease of visualization.

minutes without 95 °C heat treatment was 10^3 CFU ml^{-1} (Fig. 4D; ESI† Table S6), with different amplification times between 39 and 59 minutes. There was no amplification in milk samples spiked with 10^2 CFU ml^{-1} and LAMP assay for SEA gene with 10^1 CFU ml^{-1} concentration amplified in only one of the three replicates. The duration of heat treatment has been evaluated in combination with other DNA extraction protocols for bovine milk microorganisms.[67] We found that combining two physical preparation steps (dilution and heating) improved the assay sensitivity by an order of magnitude and decreased amplification time; this effect was consistent across cow and goat milk and three strains of *S. aureus*. Of the factors described above, there were statistically significant differences between amplification times of DNA extracts from spiked, diluted milk samples *versus* non-extracted spiked diluted milk samples ($P < 0.001$) for both genes, and for detection of SEA in heated when compared with unheated spiked diluted milk of both cows and goats. *P*-Values associated with the difference between milk from heated *versus* unheated milk

samples from different species (cow *versus* goat) are summarized in ESI† Table S7.

3.3 Optimization of developed LAMP and multiplex LAMP assays in a heat block

To translate the assay closer to a point-of-need context, the assay was transferred from a thermocycler to a heat block. Consistent with results observed on the thermocycler, on the heat block, the LOD decreased from 10^4 to 10^3 CFU ml^{-1} for goat milk samples spiked with *S. aureus* DSMZ 19041 reference strain when increasing the incubation time from 30 to 60 minutes (Table 1; ESI† Table S8). When spiked cow milk samples diluted 1:10 were tested using the optimized assay (*i.e.* incubation at 60 °C for 30 min on the heat block), the LOD was 10^5 CFU ml^{-1} for the SEA gene and multiplex LAMP assay and 10^4 CFU ml^{-1} for SEB gene (Fig. 5); this is consistent with the LODs observed on the thermocycler, except for SEA gene it was one log higher when incubated on the heat block. We also demonstrated that increasing the

Table 1 Limit of detection (LOD) of the LAMP and multiplex LAMP assay for *Staphylococcus aureus* enterotoxin A and B (SEA and SEB) genes in spiked 1:10 diluted cow milk samples at 60 °C on a heat block for 30, 45 and 60 minutes

Target		LOD of LAMP and multiplex LAMP assays for detection of SEA and SEB in spiked 1:10 diluted goat milk samples (colony forming units per ml)		
		Time (minutes)		
		30	45	60
SEA gene (*S. aureus* reference strain DSMZ 19041)		10^5	10^4	10^3
SEB gene (*S. aureus* reference strain DSMZ 19041)		10^5	10^4	10^3
Multiplex LAMP assay (SEA and SEB genes)	*S. aureus* reference strain DSMZ 19041	10^5	10^4	10^3
	S. aureus reference strain DSMZ 19044	10^5	10^4	10^3
	S. aureus reference strain DSMZ 20652	10^4	10^4	10^3
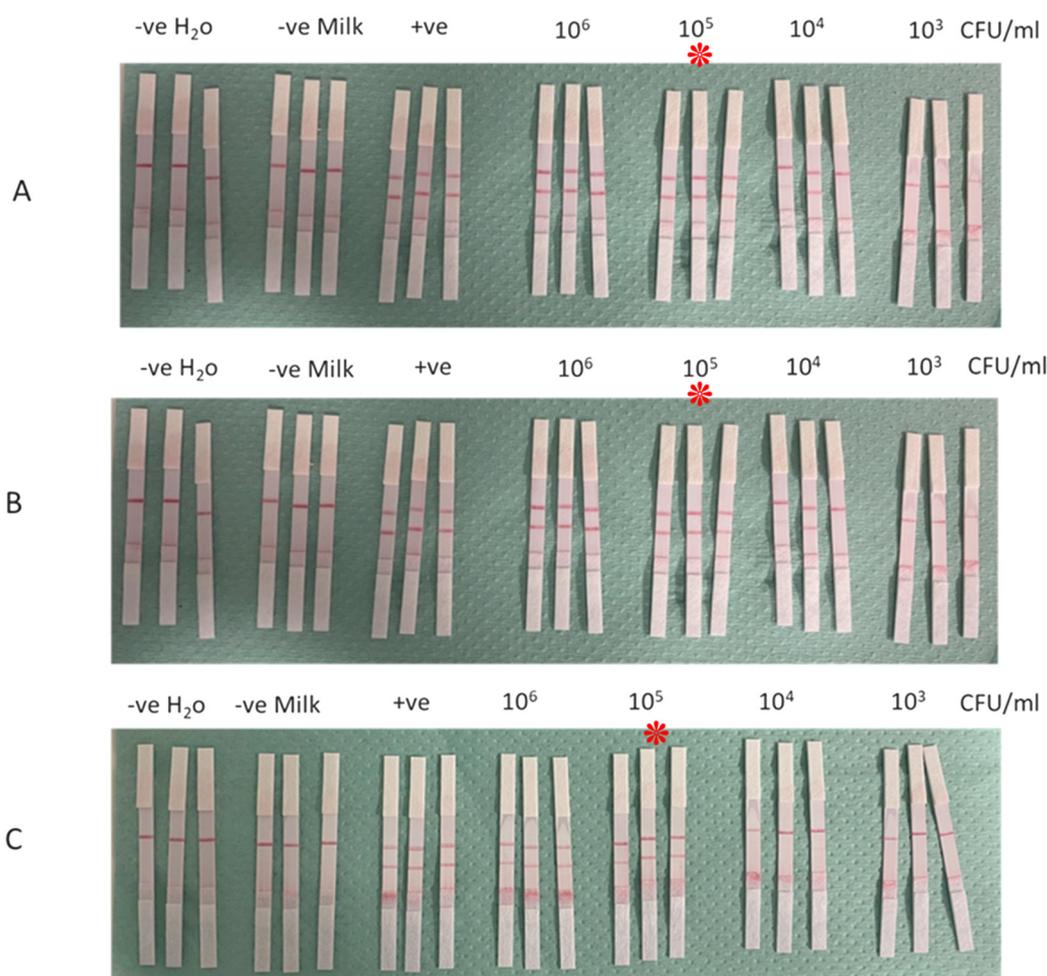
Fig. 5 Limit of detection (LOD) of loop-mediated isothermal amplification (LAMP) assay for the detection of *Staphylococcus aureus* enterotoxin A (A) and B (B) genes, as well as for the multiplex LAMP assay for both genes (C) in 1:10 diluted cow milk spiked with the cells collected from ten-fold serially diluted overnight culture of *S. aureus* DSMZ 19041 reference strain, incubated at 60 °C for 30 minutes. +ve = positive control using artificial oligonucleotide dsDNA and −ve = negative control using PCR grade nuclease free water and unspiked milk, respectively. The photos shown were cropped and edited, while original photos are in the ESI† (Fig. S10–S12), where * in each panel represent the LOD.

amplification time from 30 to 60 minutes led to the same improvement in performance as the inclusion of a thermal lysis step (Table 1). From an end-user point of view, achieving the same (clinically relevant) LOD without the need for an extra processing step that could complicate the protocol may be preferable. In summary, optimal LOD for *S. aureus* enterotoxin genes using this device can be achieved using either sample heating (lysis) or by increasing the incubation

time, thus providing two alternative assay protocols for end-users that might be more relevant for different scenarios, depending on the specific testing context, and the speed of response needed. The LOD were determined under laboratory conditions and may differ when used in the field. However, the performance is better than the recommended EU guidelines,[51] often by more than 10 times, providing room to improve.

The device and the multiplexed assay provide a positive result when either or both SEA and SEB genes are present in a sample. From a public health perspective, the presence of one or both enterotoxin genes does not change the sample's interpretation, as detecting either would lead to the same public health response. This approach is also more affordable, as it only requires the immobilization of a single capture molecule on the lateral flow strip instead of two (or the use of multiple strips as per our previous work).[45] The multiplex LAMP assay, therefore, was developed as a cumulative, non-differentiating multiplex assay for a single readout, additionally minimizing the risk of confusion for end users in field settings.

3.4 Field testing

Out of the 26 milk samples, only one individual cow sample was positive on our multiplex LAMP assay. All of the microfluidic device's negative and positive controls showed expected results (ESI† Fig. S13). After culturing all milk samples, two samples (including the one with the positive result on LAMP) showed suspect colonies with morphology consistent with Staphylococci. Biochemical testing revealed that these samples contained Gram positive, coagulase negative staphylococci (ESI† Table S9). Multiplex PCR did not detect the presence of enterotoxin A or B in these isolates (ESI† Fig. S14), suggesting the single positive outcome obtained on the device may have been a false positive. Our preliminary field validation suggests the potential ability of this test to be used in field settings; however, wider validation is required to establish robust performance metrics and usability at the point-of-need.

4. Conclusion

This study presents the development of a prototype "sample-to-answer" device for the detection of SEA and SEB genes in ruminant milk, suitable for use in low-resource settings. The developed test was rapid (results obtained within one hour), analytically sensitive and specific for the detection of *S. aureus* enterotoxin genes when using spiked samples in a laboratory setting, and robust to using commercially homogenized and pasteurised milk from different host species (cow and goat), with different *S. aureus* strains. We successfully incorporated this assay into a microfluidic device with a simple fabrication technique and an easy-to-read diagnostic result in which we combined the multiplexed test for SEA and SEB genes into a single readout, facilitating easy interpretation. By doing this, we propose that the test could greatly simplify the diagnostic testing for the most commonly occurring enterotoxin genes, especially in low resource areas, including on farms and in the case of food-borne illness emergency situations, but further work with fresh milk samples, in field settings, and with potential end-users is needed to validate the scientific and convenience characteristics of the prototype assay.

Data availability

Please note that all data associated with this publication referred to by **http://dx.doi.org/10.5525/gla.researchdata.1670** is available through the University of Glasgow's Enlighten online data repository.

Author contributions

Conceptualization, M. S., V. B. and J. R. methodology, validation and data analysis, M. S., S. C., and M. C. A. performed the experiments. All authors participated to data analysis. Designs and device fabrication, M. S. and X. Y. contributed to the fabrication. All authors contributed to design. Field testing, M. S., J. R., M. C. A. and S. C. writing – original draft, M. S. writing – review & editing, M. S. made modifications of the manuscript, V. B., T. F., J. C. and J. R. reviewed and edited, supervision, V. B., T. F. and J. R.

Conflicts of interest

No conflict of interest.

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