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Rapid and highly sensitive LAMP-CRISPR/Cas12a-based identification of bovine mastitis milk samples contaminated by *Escherichia coli*

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ABSTRACT

Mastitis is a prevalent disease affecting dairy cows, leading to significant economic losses in the dairy industry. Conventional diagnostic methods such as microbiology and PCR are expensive and time-consuming, emphasizing the need for alternative diagnostic approaches. The field of novel diagnostics is expanding rapidly due to the application of a modern molecular detection methods based on the CRISPR/Cas system. This system functions by targeting specific genetic sequences of the pathogen, including *Escherichia coli*, and detects the presence of the pathogen by employing a CRISPR RNA that complements the pathogen's genetic sequence and a Cas12a enzyme that cleaves the particular DNA sequence. In this paper, we present a novel pathogen detection technology that combines the loop-mediated isothermal amplification (LAMP) reaction and Cas12a collateral activity. We have successfully developed a rapid and precise method for identifying *E.coli* genomic DNA using the LAMP-Cas12a technology, which exhibits high analytical sensitivity within an hour, detecting as low as 1.3×10^1 copies of target DNA. This technology also has the ability to differentiate *E. coli* from other prevalent mastitis pathogens such as *Staphylococcus aureus* and *Streptococcus agalactiae*. Furthermore, we employed a novel MbCas12a nuclease, which demonstrated excellent diagnostic performance in our study when identifying *E.coli* isolates isolated from bovine mastitis milk samples. The development of such new methods has the potential to expand agricultural tools for use in point-of-care (POC) diagnostics.

1. Introduction

The progress of agriculture, particularly animal husbandry, is closely linked to the prevalence of farm animal diseases. With the expansion of large-scale and intensive farming, mastitis in cattle, including cows, has become an increasingly common problem that significantly impacts productivity and results in substantial economic losses [1–3]. Mastitis is an infectious disease characterized by inflammation of the udder in animals, resulting from infection of the mammary glands. The disease adversely affects animal health and presents symptoms such as udder inflammation and swelling, changes in the color and texture of the milk, elevated body temperature, and general weakness [4]. These symptoms arise from an infection caused by pathogenic and opportunistic bacteria, including *Streptococcus* spp., *Staphylococcus* spp., *E.coli*, *Enterococcus* spp., *Klebsiella* spp., and other related species [5]. Consequently, the deterioration in milk quality and dairy products resulting from mastitis not only hinders animal husbandry and causes economic losses, but also poses a potential to risk of widespread food poisoning in humans, potentially leading to fatalities.

The eradication of mastitis has emerged as a critical issue in the ongoing development of animal husbandry and the improvement of milk production quality. Timely diagnosis is the initial step in addressing this issue, as it can prevent the progression of the disease in its early stages, minimizing losses. Various methods are available for diagnosing mastitis, including visual examination of the udder and milk, as well as laboratory-based approaches such as bacteriological analysis of milk and PCR diagnostics [6]. However, traditional diagnostic methods may not always be effective since inflammatory processes can occur not only

Abbreviations: CRISPR, clustered regularly interspaced short palindromic repeats.

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with severe clinical symptoms but also in a latent form, making it nearly impossible to visually detect the presence of the disease [7]. Moreover, conventional laboratory methods can be time-consuming, expensive, and may lack the required sensitivity and specificity for accurate diagnosis.

Consequently, early, rapid, and cost-effective diagnosis plays a critical role in addressing the aforementioned problem. The development of new diagnostic techniques based on CRISPR/Cas technology holds great promise in meeting these requirements. CRISPR/Cas is a genome-editing tool derived from the bacterial immune system's mechanism against viruses [8]. This mechanism enables bacteria to defend themselves by capturing and preserving viral DNA in CRISPR loci [9]. Beyond its role in bacterial immunity, the CRISPR/Cas mechanism can be harnessed for specific diagnostic purposes. Upon viral re-infection, the CRISPR loci are activated, leading to the production of guide RNAs (crRNA). That, the Cas protein, in conjunction with crRNA, cleave the viral DNA, effectively destroying it. The recently discovered collateral activity of the Cas12a enzyme [10], enables accurate and utilization of this technology in diagnostics, without the need for highly specialized personnel or expensive equipment. This has the potential to reduce costs and increase the accessibility of diagnostics in the modern world.

The technology relies on the recognition of the protospacer adjacent motif (PAM) sequence on the amplified DNA region by the Cas enzyme. Upon recognition, a complex is formed between the Cas enzyme and the crRNA targeting the specific DNA sequence, leading to cis activity with subsequent DNA cleavage. The detection of the target triggers conformational changes in the Cas enzyme [11,12], which activate its *trans*--activity, resulting in sequence-nonspecific single-stranded DNA cleavage. This property is harnessed as a diagnostic tool, with detection being achieved, for example, through the emission of a fluorescent signal [13–18].

In this study, we utilized CRISPR/Cas-based diagnostics integrated with the LAMP (Loop-mediated isothermal amplification) platform to identify *E.coli* as one of the causative agents of mastitis. Furthermore, we explored the potential of a novel Cas effector homologue, MbCas12a from *Moraxella bovis*, as an alternative to the commonly available analogues in CRISPR/Cas diagnostics. This approach expands the range of Cas12a enzymes applicable for diagnostic applications [19–22].

2. Materials and methods

2.1. Oligonucleotides

The following oligonucleotides were used in the work (Table 1).

2.2. Cloning, expression and purification of MbCas12a

Genomic DNA from *Moraxella bovis* was extracted using the Genomic DNA Purification Kit (Promega, USA). The reference gene sequence for the *Moraxella bovis* cas12a gene was obtained from the published genome sequence NZ_CP087840.1 (range: 623690–627475). The specific oligonucleotide primers used for gene amplification are provided in the list of oligonucleotides.

cas12a gene was amplified from genomic DNA of *Moraxella bovis* using MbovisCas12aFW and MbovisCas12aRV primers and the resulting amplicon was cloned into pET28c(+) vector at *NdeI/BamHI* sites. The recombinant protein MbCas12a was purified from *E.coli* ArcticExpress (DE3) cells transformed with the pET28c(+)/MbCas12a plasmid. Once cells reached an optical density of 0.6 at 600 nm, they were induced with 0.5 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) and incubated at room temperature with shaking at 100 rpm for 16 h. Following induction, the cells were harvested at +4 °C by centrifugation at 6000×*g* for 7 min [23]. The cell pellet was resuspended in a buffer containing 20 mM Tris-HCl pH 8.0, 150 mM NaCl, and a cocktail of protease inhibitors (Roche Diagnostics). Subsequently, the cells were incubated with lysozyme (3 mg/ml) for 20 min at room temperature and subjected to ultrasound treatment (50 kHz) in a pulsed mode on ice. The resulting lysate was centrifuged at +4 °C for 1 h at 40,000×*g*. MbCas12a protein was purified using immobilized metal affinity chromatography (IMAC) on a HisTrap HP column (Cytiva), with a linear imidazole gradient from 50 mM to 500 mM. The fractions containing the target protein were combined and loaded onto a HiTrap Heparin HP column (Cytiva) in manual mode. The eluate was purified and fractionated using a linear NaCl gradient from 50 mM to 1000 mM on an FPLC AKTA Purifier 10 (GE Healthcare). The fractions containing the recombinant protein were stored at −20 °C in 50% glycerol. The identity of the recombinant protein was confirmed using reversed-phase C18 liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis, as previously described [24].

2.3. Design of crRNAs

To design the crRNA, we obtained data from various *E.coli phoA* gene sequences available in the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/genome). DNA fragments from the chromosomal sequences (GenBank) of these genes were chosen as the source of the DNA template. Candidate target regions of approximately 20–100 nucleotides in size on the pathogen chromosomes were identified. The design of the crRNA was guided by the presence of protospacer adjacent motif (PAM) regions. Considering the specificities of the amplification products, we selected two different sites for crRNA design.

2.4. Synthesis of crRNAs

crRNAs were synthesized using the HiScribe ™ T7 Quick High Yield RNA Synthesis Kit (New England Biolabs). To prepare the DNA template for *in vitro* transcription, synthetic DNA oligonucleotides of different lengths containing the double-stranded region of the T7 promoter preceding the transcription sequence were utilized. The minimum T7

Table 1
Oligonucleotides.

Method	Oligonucleotide	Sequence (5'→3')
PCR	MbovisCas12aFW	GGCAGCCATATGTTATTTCAAGAGTTTACCC
	MbovisCas12aRV	CAAGGATCCTTAGCGGTTTTGAGCGAAG
In vitro transcription	crRNA-PAM1-compl	**AGCGCACTGGCATATTGCCGTCAG**ATCTACAAACAGTAGAAATTCCCTATAGTGAGTCGTATTAGAATT
	crRNA-PAM2-compl	**AATGCGGTGACGGAAGCGAACCAG**ATCTACAAACAGTAGAAATTCCCTATAGTGAGTCGTATTAGAATT
	crRNA-SHORT	AATTCTAATACGACTCACTATAGGG
LAMP	F3-phoA	TGTCATTACGTTGCGGATT
	B3-phoA	CTTTGCTGAAACGGCAAC
	FIP-phoA	CTGACGGCAATATGCCAGTGGTCGATATTGCCGTGGTACG
	BIP-phoA	GTTCGCTTCCGTCACCGCGCGTGGTTATCAGTTGGT
	LF-phoA	GCTGGCAAGGACCGAAAG
	LB-phoA	TTCAGTGAGGCAGCATCG
Fluorescence	ssDNA Reporter	FAM-TTATT-BHQ-1
LFA	ssDNA LFA Reporter	FAM-TTATTATT-Biotin

promoter sequence, 5′-TAATACGACTCACTATAGGG, was employed. To generate the template, a short oligonucleotide (10 μM) was annealed with its corresponding complementary oligonucleotide (10 μM) at 75 °C for 3 min and then cooled to room temperature for 1 h. The resulting duplex oligonucleotide served as the template for *in vitro* transcription.

The transcribed RNA was purified using the Monarch® RNA Cleanup kit (New England Biolabs, MA, USA) according to the manufacturer's protocol, with the inclusion of DNase I treatment to remove any remaining template DNA. The purified RNA was quantified using a Nanodrop spectrophotometer (Thermo Scientific) and diluted to working concentrations with DEPC-treated water, ensuring the absence of nucleases. The eluted RNA was either used immediately or stored at −80 °C.

2.5. Construction of plasmid with positive control

A plasmid containing a positive control was constructed as follows. The *phoA* gene was amplified using Phusion DNA polymerase through the polymerase chain reaction. The resulting 253 bp PCR product was confirmed to be positive and was cloned into a genetic construct using the CloneJET PCR Cloning Kit (Thermo Scientific). The resulting plasmid was sequenced to confirm the absence or presence of mutations. This positive control was used to optimize the LAMP reaction and assess the sensitivity of the assay.

2.6. LAMP reaction conditions

LAMP reactions were performed in a final volume of 25 μl, 1.6 μM each of the FIP and BIP primers, 0.2 μM each of the F3 and B3 primers, 0.8 μM each of the LF and LB primers, 1.4 μM each of the dNTPs, 0.8 M betaine, 1 × Isothermal amplification buffer, 6 mM $MgSO_4$, *Bst* 4U DNA polymerase (New England Biolabs) and the specified amount of DNA template. After incubation at 62 °C for 45 min, the reaction was stopped by heating at 95 °C for 5 min.

2.7. CRISPR/Cas12a-LAMP fluorescence assay

To assemble the MbCas12a and crRNA complexes, 1 μM of MbCas12a (obtained from laboratory stock) and 1 μM of crRNA were incubated at 25 °C for 15 min. The *trans*-cleavage reaction of Cas12a was conducted in a 30 μl volume containing NEB 2.1 buffer (New England Biolabs), 100 nM crRNA, 100 nM MbCas12a, 5 μM reporter molecule, and 3 μl of LAMP amplification products. The reaction mixture was incubated at 37 °C for 30 min. The reaction results were visualized using a Vilber Lourmat transilluminator (France) with the naked eye at a wavelength of 320 nm. Images were captured using a smartphone camera and saved for further analysis.

2.8. Real-time fluorescence read-out

The real-time fluorescence signal was measured using a CFX96 Real-Time System C1000 Touch Thermal Cycler, and fluorescence readings were recorded every minute over a 30-min period.

2.9. Evaluation of limit of detection and specificity of LAMP-Cas12a detection

To assess the sensitivity of the LAMP assay, we conducted reactions using ten-fold serial dilutions of both genomic DNA and plasmid DNA. The plasmid DNA, containing the *phoA* gene fragment sequence, was diluted from 1.4×10^{12} to $1.4 \times 10^{\circ}$ copies per reaction. Likewise, genomic DNA was diluted from 1.3×10^{9} to 1.3×10^{2} copies per reaction.

To evaluate the specificity of LAMP-Cas12a detection, the assay was tested against non-target bacterial strains, including *Staphylococcus aureus* and *Streptococcus agalactiae*, which are commonly found in milk samples.

2.10. Evaluation of clinical samples

E.coli samples were isolated from milk samples obtained from household cows exhibiting clinical symptoms of mastitis. All isolates were grown in monoculture and confirmed using both microbiological methods and the matrix-assisted laser desorption ionization (MALDI) Biotyper microbial identification system (Bruker) [25]. The genomic DNA of the isolates was extracted using the Monarch® Genomic DNA Purification Kit. Following extraction, the genomic DNA of the isolates was used as a template for performing the LAMP reaction.

2.11. LAMP-CRISPR/Cas12a-based lateral flow assay

In this study, we examined the utilization of the MbCas12a enzyme in combination with a FAM-TTATTATT-Biotin reporter to cleave LAMP products. The reaction buffer included 1x NEB Buffer 2.1, 500 nM Cas12a, 1 μM crRNA, and 1 μM single-stranded DNA reporter, with 2 μl of LAMP product added to the mixture. The reaction was conducted at 37 °C for 30 min. Subsequently, the cleaved products were applied to a lateral flow strip for 3 min, followed by immersion in 100 μl of HybriDetect buffer (Milenia HybriDetect, Milenia Biotec, Gieβen, Germany) and mixed for 5 min at room temperature for analysis.

2.12. Reagents

All reagents were from New England Biolabs, Sigma and Thermo Scientific in the molecular biology category. The Cas12a enzyme from *Moraxella bovis* was produced in our own laboratory.

2.13. Statistical analysis

Statistical analysis was conducted using a GraphPad Prism version 8.0. software (GraphPad Software Inc., San Diego, CA, USA). Values are presented as means ± standard deviation (SD).

3. Results

3.1. The principle of LAMP-CRISPR/Cas12a assay

Loop-mediated isothermal amplification (LAMP) is a simple and rapid method of DNA amplification that can be used as a pre-amplification method for CRISPR/Cas-based diagnostics. This method utilizes a set of four to six primers that specifically target multiple regions of the target DNA sequence, resulting in highly specific and sensitive amplification of the target sequence [26].

Cas12a-based diagnostics utilize the CRISPR/Cas system for target-specific detection of nucleic acids. Cas12a, an RNA-guided endonuclease, upon activation by a specific target DNA sequence, cleaves nearby single-stranded DNA (ssDNA) molecules, resulting in the release of a fluorescent signal. However, Cas12a is sensitive to the quantity of the target DNA within the sample, and therefore, a pre-amplification step may be required to enhance the assay's sensitivity.

Incorporating LAMP as a pre-amplification method for Cas12a-based diagnostics offers several advantages. LAMP is a rapid and easy method that does not require specialized equipment or expertise. Moreover, LAMP can amplify very low quantities of target DNA, making it a highly sensitive method. By combining LAMP with Cas12a-based diagnostics, it is possible to increase the sensitivity of the assay and minimize the occurrence of false negatives.

The LAMP amplification products were required to contain the target sequence and PAM region. Specifically, the sequence of the *phoA* gene, a housekeeping gene that codes for bacterial alkaline phosphatase, was selected as the target for this study. This gene is commonly used as a diagnostic marker for the identification of *Escherichia coli* [27–29]. In

this work, the primers designed by Griffioen, K., et al. were used for loop isothermal amplification with minor modifications [30]. We selected two targets (crRNAs – PAM1 and PAM2) with identical PAM sequences (TTTG) to ensure a fair comparison of enzyme activity and to exclude potential differences in the kinetics of Cas12a-crRNA/target complex formation. The principle of LAMP-CRISPR/Cas12a Assay is shown in Fig. 1.

3.2. Evaluation of limit of detection

To assess the sensitivity of the developed method, experiments were conducted to determine the limit of detection (Fig. 2).

Fig. 2A shows the amplification plot of LAMP products generated from dilutions of the positive control plasmid, while Fig. 2B shows the detection plot of *trans*-cleavage activity by MbCas12a using the LAMP products as targets. The plot demonstrates a clear signal above the background level, indicating successful detection of the target sequence. The limit of detection, determined to be $1.4 \times 10^{\circ}$ copies per reaction, signifies the high sensitivity of the LAMP-Cas12a method for detecting the *phoA* gene locus.

After optimizing LAMP reaction conditions for plasmid DNA, the same primer combination and optimized conditions were applied to isolated *E.coli* genomic DNA. To assess sensitivity, genomic DNA was diluted in a 10-fold manner, resulting in a range of final concentrations from 1.3×10^{9} to $1.3 \times 10^{\circ}$ copies of genomic DNA used as DNA template. The agarose gel electrophoresis demonstrates that the LAMP reaction proceeds effectively up to a dilution of 1.3×10^{1} copies of genomic DNA (Fig. 2C). Similar to the experiments conducted with plasmid DNA, the products of the LAMP reaction using genomic DNA were used to determine the sensitivity of *trans*-cleavage activity. The limit of detection (LoD) for *trans*-cleavage activity was found to be 1.3×10^{1} copies of the *phoA* gene locus (Fig. 2D). It is important to note that the decrease in fluorescence signal with decreasing concentration of genomic DNA used in the isothermal amplification is expected since the amount of target DNA available for detection is reduced, resulting in less cleavage of the reporter oligonucleotide by the Cas12a enzyme. Hence, the fluorescence signal level serves as an indicator of the quantity of target DNA present in the sample.

The data presented in Fig. 2B and D indicates that at the final dilutions, the fluorescence level using PAM1 is higher compared to PAM2. Several factors could contribute to this observation, including variations in the efficiency of Cas12a-crRNA/target complex formation, differences in the kinetics of target accumulation, or differences in the accessibility of the target sequences to the Cas12a-crRNA complex.

In this study, we utilized two targets (target 1 and target 2) for Cas12a to assess the potential impact of utilizing the same PAM region sequence. Visual examination of the fluorescence level of *trans*-cleavage activity, using LAMP products derived from plasmid DNA, revealed a noticeable difference in fluorescence intensity between PAM1 and PAM2. This divergence could potentially be attributed to the formation of distinct LAMP product structures, which may influence the ability of the Cas12/crRNAPAM1 complex to more readily form a complex with the target DNA in comparison to the Cas12/crRNAPAM2 complex.

Thus, in addition to visual inspection, we also evaluated the results of *trans*-cleavage activity using fluorescence signal measurements with PAM1-2 crRNA (Fig. 3).

As can be seen, crRNA shows high analytical sensitivity to the target. The Cas12a exhibits the capability to detect target sequences even at very low concentrations, with a limit of detection as low as 1.4×10^{1} copies of plasmid DNA.

Moreover, we performed a sensitivity analysis using *E.coli* genomic DNA. The findings revealed that Cas12a effectively detects LAMP amplification products, even at an initial genomic DNA concentration as low as 1.3×10^{1} copies of the *phoA* gene. This highlights the high analytical sensitivity of our CRISPR-based LAMP detection technology. The detection sensitivity was determined by agarose gel electrophoresis and measurement of the fluorescence signal. It was observed that the fluorescence signal level decreased proportionately with decreasing genomic DNA concentration used in isothermal amplification, thereby confirming the limit of detection.

3.3. Real-time fluorescence read-out

In both scenarios, using both PAM1 and PAM2 crRNA, a significant

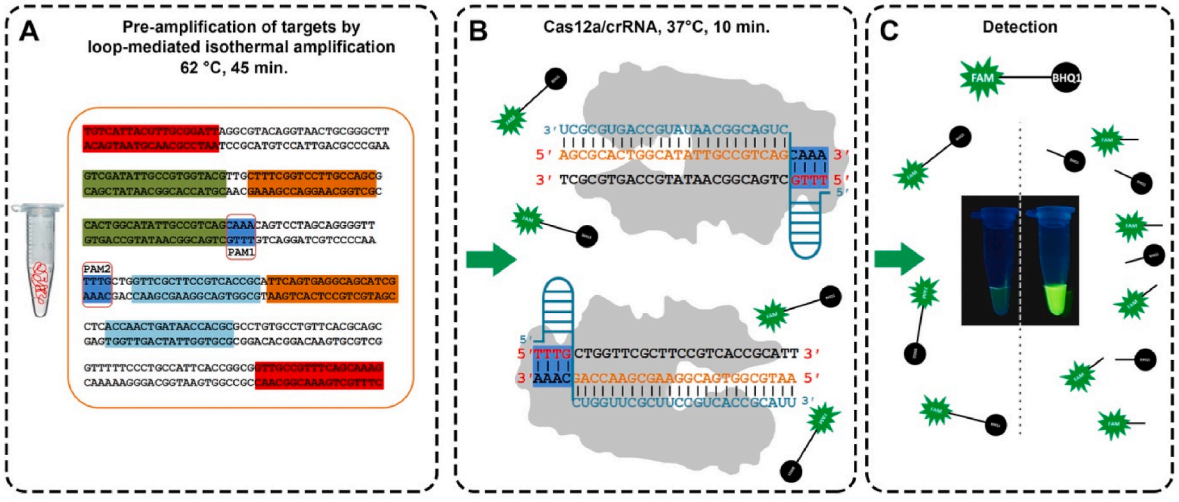

Fig. 1. Schematic representation of the CRISPR-based LAMP dual-target detection technology for *E.coli*. **(A)** Pre-amplification step involves the LAMP reaction targeting the *phoA* gene locus. The target contains two sites for the Cas protein with the same sequence of PAM – TTTG (two squares PAM1 and PAM2). Different colors indicate complementary regions of LAMP oligonucleotides: FIP (green) and BIP (blue), F3 and B3 (red), and loop primers (orange). Orange boxes highlight two PAM sites - TTTG; **(B)** Cas12a detects accumulated targets through *cis*-activity and forms Cas12a/crRNA/dsDNA complexes. After the assembly of the complex, the Cas12a enzyme undergoes a conformational alteration, subsequently leading to its *trans*-cleavage activity and cleavage of fluorescent single-stranded DNA (ssDNA). The length of the complex is 24 nt. The reaction includes fluorescently labeled short oligonucleotides (reporters) which act as an indicator of *trans*-cleavage activity. The reporters are labeled with FAM fluorescent label at the 5′ end and BHQ1 quencher at the 3′ end; **(C)** Detection of reporter digestion results with fluorescence emission (right side). In the absence of a target, no cleavage of the fluorescent reporter occurs (left side). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

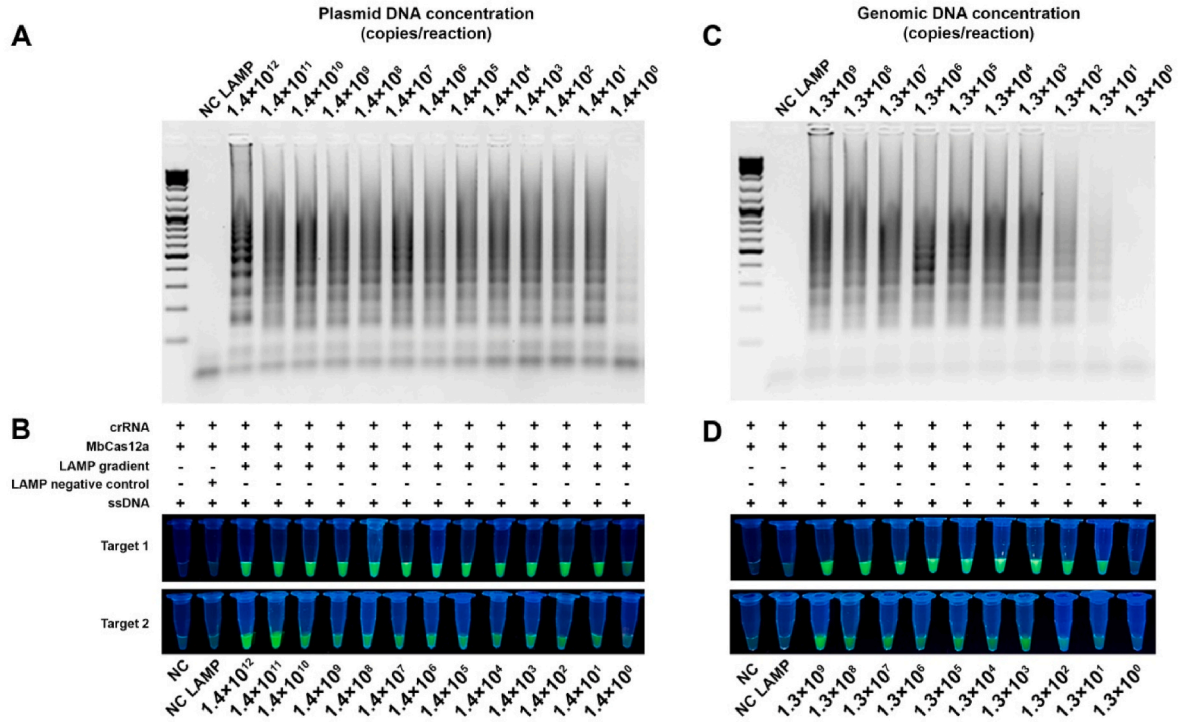

Fig. 2. The analytical sensitivity evaluation of CRISPR/Cas12-LAMP for *E.coli phoA* gene detection was conducted and the results are as follows: **(A)** An agarose gel electrophoresis image of LAMP products with serially 10-fold diluted positive plasmid containing the *phoA* gene as the target; **(B)** The results of *trans*-cleavage activity on the LAMP product with plasmid DNA using PAM1 crRNA (target 1) and PAM2 crRNA (target 2) after 30-min incubation. The image was captured under UV light using a smartphone camera; **(C)** An agarose gel electrophoresis image of LAMP products with serially 10-fold diluted genomic DNA; **(D)** The results of *trans*-cleavage activity on the LAMP product with genomic DNA using PAM1 crRNA (target 1) and PAM2 crRNA (target 2) after 30-min incubation. The image was captured under UV light using a smartphone camera. NC LAMP – negative control, LAMP reaction without matrix DNA. NC - negative control, *trans*-cleavage activity of MbCas12a without pre-amplification product.

difference in fluorescence level was detected by the instrument. Interestingly, PAM1 crRNA consistently generated higher fluorescence values in both plasmid DNA and genomic DNA samples. To further investigate the detection capability of PAM1 crRNA, the generation of fluorescence signal was evaluated over a 30 min period using serially diluted concentrations of template DNA, specifically with the utilization of crRNA (Fig. 4).

3.4. Evaluation of specificity and LAMP-Cas12a detection on clinical specimens

To assess the specificity of the LAMP-Cas12a assay, a BLAST analysis was initially conducted to verify the specificity of the target sequence for *E.coli*. The analysis revealed high specificity, indicating that the target sequence did not exhibit significant homology to other bacterial species (data not shown). Fig. 5 shows the results obtained from testing the specificity of the CRISPR/Cas12a/LAMP technology using genomic DNA of *Staphylococcus aureus* and *Streptococcus agalactiae*.

The specificity of the Cas12a-LAMP assay for detecting *E.coli* was evaluated, and the assay exhibited a high level of specificity, even when tested against other mastitis-associated pathogens such as *S. aureus* and *S. agalactiae*, as shown in Fig. 5. Notably, the assay successfully differentiated *E.coli* from other mastitis pathogens, indicating its high specificity. The fluorescent signal obtained from the *Escherichia coli* reaction tube was notably higher compared to the other tubes, suggesting the excellent specificity of the primers used for targeting the *E. coli* sequence. These results also suggest that the isothermal reaction amplification products are efficiently recognized by the Cas12a/crRNA complex, leading to the generation of a robust fluorescent signal.

3.5. Verification of the LAMP-CRISPR/Cas12a method using mastitis milk samples

Next, we tested the clinical isolates of *E.coli* using the LAMP-Cas12a assay. The results showed that all 13 isolates of *E.coli* were positive for the presence of the *phoA* gene (Fig. 6), confirming the sensitivity and specificity of the assay. The results for the clinical samples were found to be comparable to those of the plasmid and genomic DNA from the optimization experiments.

The results from Fig. 6 demonstrate that the LAMP-CRISPR/Cas12a technology successfully identified the DNA of all clinical isolates, in contrast to the negative control. Notably, a distinct fluorescent signal was observed as early as 10 min.

3.6. LAMP-Cas12a detection of clinical samples with lateral flow assay

In order to expand the detection capabilities beyond fluorescent signals, the detection reaction in this study was conducted using the lateral flow assay method (Fig. 7).

During the LAMP reaction, the DNA polymerase amplifies the target sequence, leading to the production of abundant specific double-stranded DNA (dsDNA) products. The Cas12a/crRNA complex is activated upon detection of the amplified dsDNA and cleaves the ssDNA probe in non-specific manner. The accumulation of cleaved reporter molecules results in the appearance of a visible line at the test line (Fig. 8).

Following the manufacturer's protocol, the LAMP reaction products from the 13 isolates were applied to LFA (Lateral Flow Assay) strips. The results showed that all 13 clinical samples were successfully identified using the LAMP-CRISPR/Cas12a-based lateral flow assay. The effectiveness of the MbCas12a enzyme and the FAM-TTATTATT-Biotin

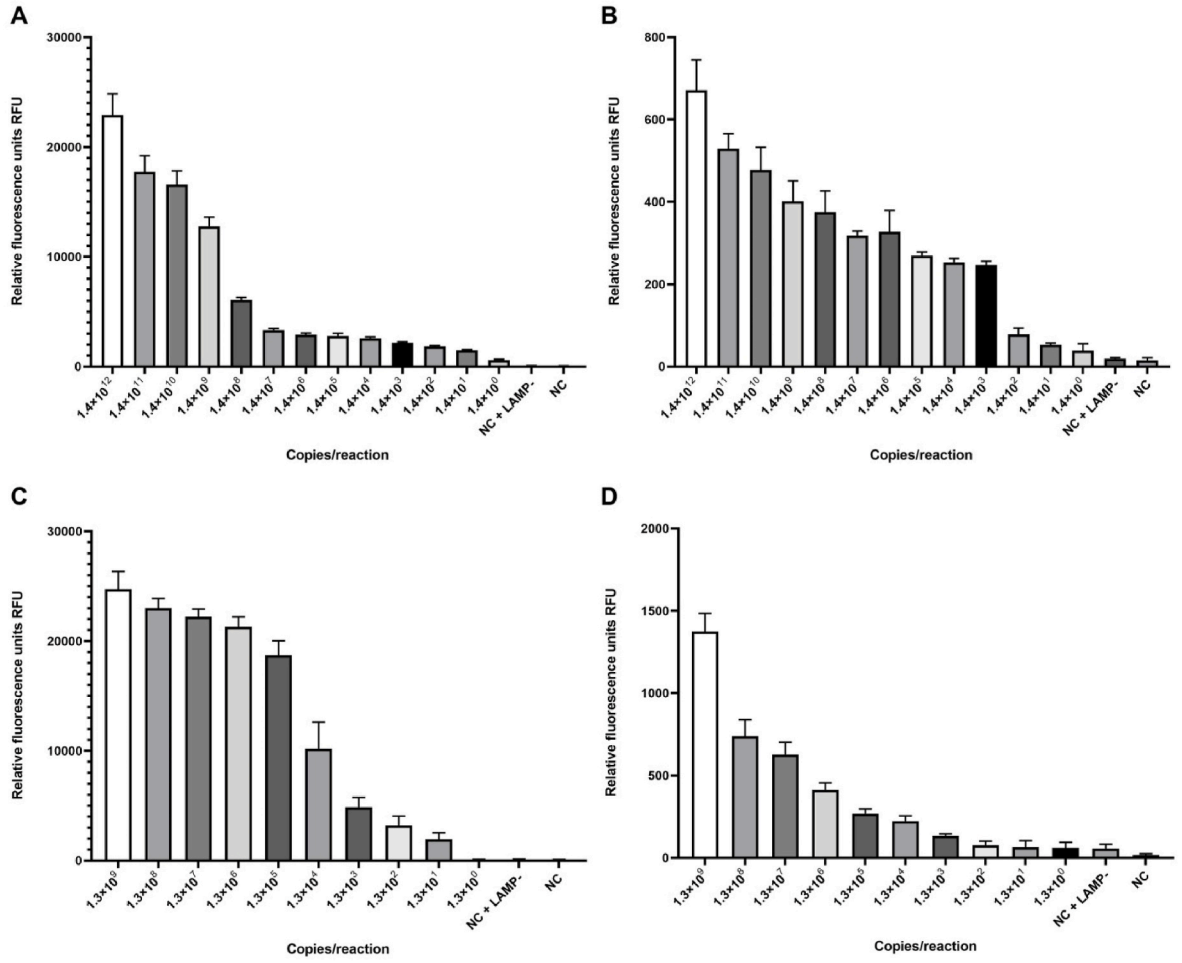

Fig. 3. Quantification of fluorescence intensity. **(A)** The fluorescence signal level of *trans*-cleavage activity using amplification products from a gradient of diluted plasmid DNA for target 1 (PAM1); **(B)** The fluorescence signal level of *trans*-cleavage activity using amplification products from a gradient of diluted plasmid DNA for target 2 (PAM2); **(C)** The fluorescence signal level of *trans*-cleavage activity using amplification products from a gradient of diluted genomic DNA for target 1 (PAM1); **(D)** The fluorescence signal level of *trans*-cleavage activity using amplification products from a gradient of diluted genomic DNA for target 2 (PAM2). Additionally, "NC + LAMP-" represents the CRISPR/Cas12a reaction with negative LAMP product, and "NC" represents the CRISPR/Cas12a reaction without amplification product.

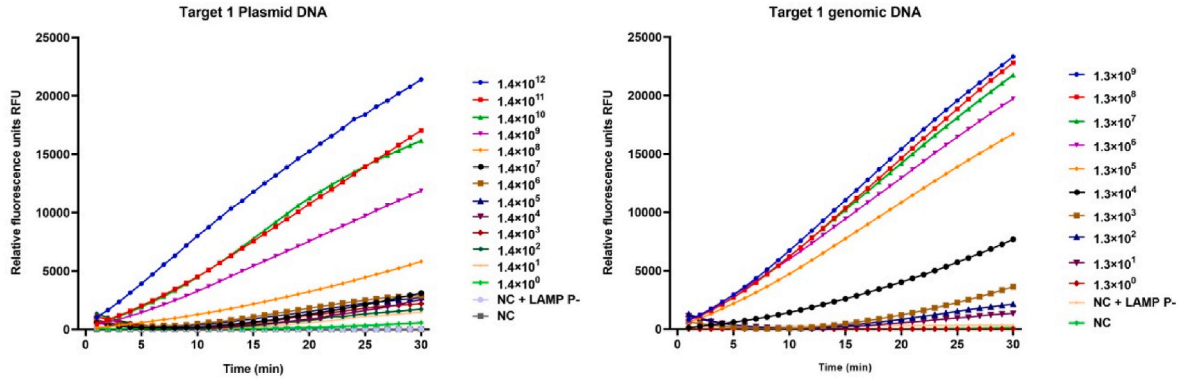

Fig. 4. Sensitivity of LAMP-CRISPR/Cas12a Assay. "NC + LAMP-" - represents the CRISPR/Cas12a reaction with negative LAMP product. "NC" - represents the CRISPR/Cas12a reaction without amplification product.

reporter in cleaving LAMP products was demonstrated through the successful application of cleaved products to a lateral flow strip for analysis. These findings demonstrate the utility of this approach for clinical sample detection and analysis.

4. Discussion

The CRISPR/Cas system has shown great promise in revolutionizing the field of molecular diagnostics. As a powerful gene editing tool, it can be repurposed to detect specific genetic sequences, including those

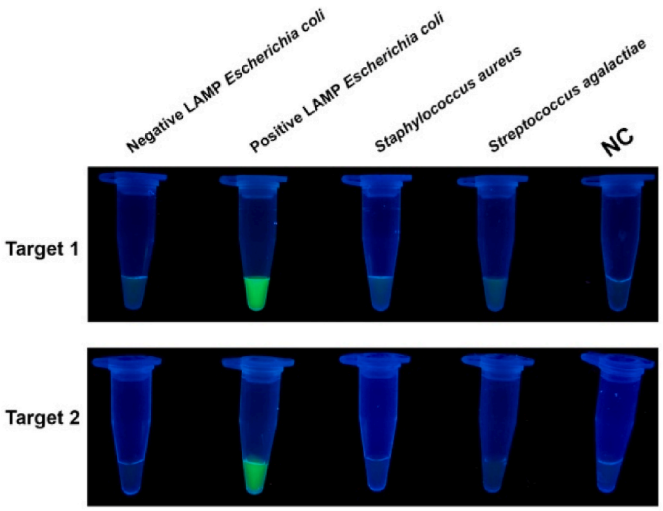

Fig. 5. Specificity of the LAMP-Cas12a assay for target 1 and target 2 using genomic DNA of mastitis' pathogens *Staphylococcus aureus* and *Streptococcus agalactiae*. Negative LAMP *Escherichia coli* – represents the CRISPR/Cas12a reaction with negative LAMP product using genomic DNA of *Escherichia coli*. Positive LAMP *Escherichia coli* - represents the CRISPR/Cas12a reaction with positive LAMP product using genomic DNA of *Escherichia coli*. *Staphylococcus aureus* - represents the CRISPR/Cas12a reaction with negative LAMP product using genomic DNA of *Staphylococcus aureus*. *Streptococcus agalactiae* - represents the CRISPR/Cas12a reaction with negative LAMP product using genomic DNA of *Streptococcus agalactiae*. "NC" - represents the CRISPR/Cas12a reaction without amplification product.

associated with bacterial pathogens. To develop an alternative diagnostic for mastitis based on the CRISPR/Cas system, the first step is to identify the unique genetic sequences specific to the mastitis-causing bacteria like *Escherichia coli*. This can be achieved by designing of crRNA targeting pre-amplification products obtained from bacterial genome isolated from infected milk samples. Once the genetic sequences have been identified, the CRISPR/Cas system can be employed to target and detect these sequences. The system functions by utilizing a crRNA to target a specific genetic sequence, which is subsequently cleaved by the Cas12a enzyme, resulting in the release of a fluorescent signal that can

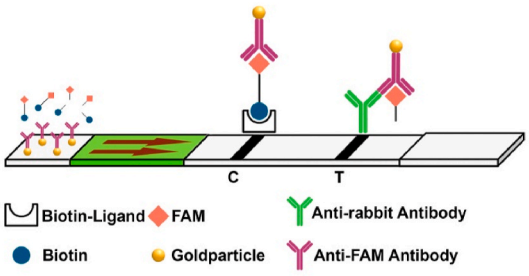

Fig. 7. The working principle of lateral flow assay. Lateral flow assay is based on the principles of the gold nanoparticle-based colorimetric detection. It involves the use of a reporter molecule, a single-stranded DNA (ssDNA) probe labeled with FAM and biotin at the 5′ and 3′ ends, respectively. The ssDNA probe migrates along the lateral flow strip towards the control line. The control line contains immobilized ligand (streptavidin) that binds to uncleaved reporter, forming a visible colored line at the control line (negative result). A positive result is indicated by the appearance of a visible line at the test line, indicating the presence of the target sequence in the original sample. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

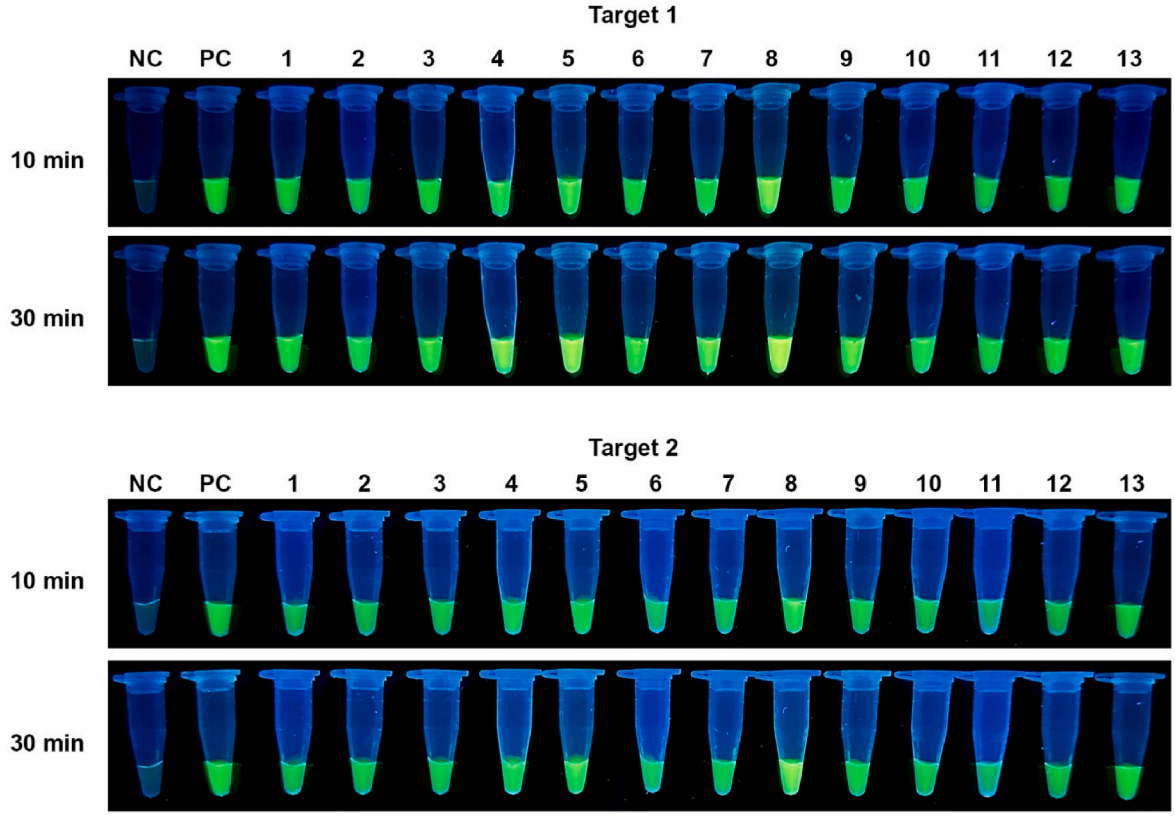

Fig. 6. Evaluation of the LAMP-Cas12a assay on clinical isolates of *E.coli*. CRISPR/Cas12a reactions on targets 1 and 2 after 10 and 30 min "NC" represents negative control, while "PC" represents positive control.

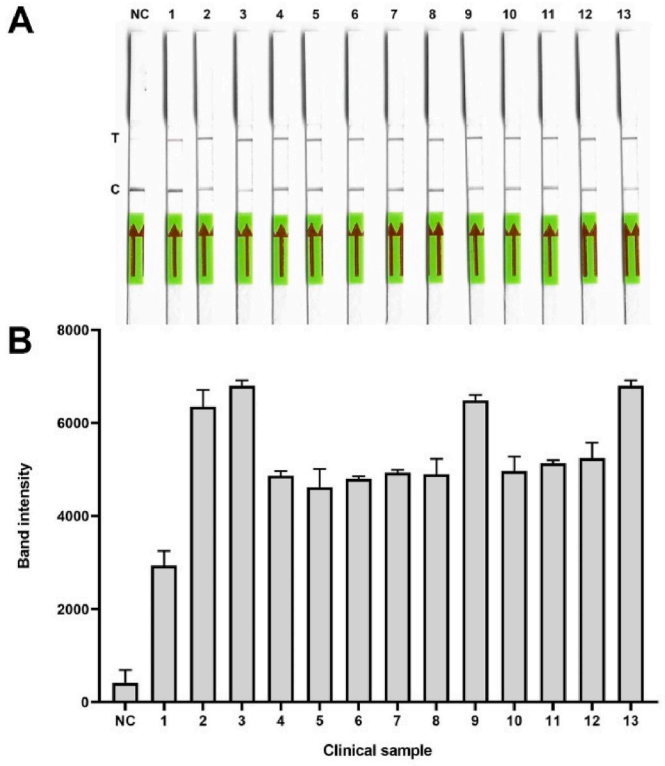

Fig. 8. (A) Detection of 13 clinical isolates of *E.coli* with the LAMP-CRISPR/Cas12a-based lateral flow assay. **(B)** Graphical representation.

be detected. Compared to traditional diagnostic methods such as microbiology and PCR diagnostics, the CRISPR/Cas system offers several advantages. It is faster, more sensitive, and more specific, enabling earlier detection of mastitis and more accurate identification of the causative agent. Furthermore, the system can be easily adapted to detect other bacterial pathogens that cause mastitis such as *Staphylococcus aureus* and *Streptococcus agalactiae*.

In this study, we have developed a technology for the detection of *E. coli*, one of the main causative agents of bovine mastitis, with high analytical sensitivity, capable of detecting as low as 100 copies of the gene. Moreover, for the diagnostic application of this technology, it is crucial to determine its cross-reactivity with other mastitis pathogens such as *Staphylococcus aureus* and *Streptococcus agalactiae*. To assess the specificity of the technology, we conducted tests using genomic DNA of these pathogens under the same conditions optimized for *E.coli* detection. The results demonstrated that the LAMP/Cas12a technology can specifically detect *E.coli* without exhibiting non-specific *trans*-cleavage activity of Cas12a. The obtained data on analytical sensitivity and specificity indicate that the presented pathogen detection technology is comparable to traditional nucleic acid amplification-based methods, such as the polymerase chain reaction.

Using LAMP as a pre-amplification method for Cas12a-based diagnostics is a fast, easy, and highly sensitive approach that can enhance the accuracy of nucleic acid detection assays. This powerful combination has the potential to revolutionize the field of molecular diagnostics and advance the detection of diverse diseases and pathogens.

It is worth noting that this study utilized a novel Cas12a homologue from *Moraxella bovis*. The results demonstrated that this homologue is a promising alternative to previously characterized Cas12a homologues. One notable advantage of utilizing the MbCas12a homologue is its greater variability in the PAM sequence. A PAM search assay was performed, revealing that MbCas12a exhibited robust cis activity with various PAM sequences, including TTTA, TCTA, TTCA, TCCA, CTTA, CCTA, and CCCA, in addition to the classic TTTG sequence. This expanded range of target detection provides a broader applicability for the technology [31]. Similar findings have been previously reported for the closely related homologue from *Moraxella bovoculi* [32]. The exploration of alternative homologues like MbCas12a is essential for enabling rapid, reliable, and cost-effective pathogen detection in challenging environments, particularly those related to food biosafety [33].

Ethics approval

Not applicable.

Credit author statement

Aisha Shaizadinova: Methodology; Software; Validation; Visualization; Writing - original draft; Meruyert Amanzholova: Methodology; Software; Validation; Visualization; Writing - original draft; Saveliy Kirillov: Methodology; Software; Validation; Visualization; Writing - original draft; Aitbay Bulashev: Data curation; Formal analysis; Funding acquisition; Investigation; Writing - original draft; Writing - review & editing; Sailau Abeldenov: Data curation; Formal analysis; Funding acquisition; Investigation; Project administration; Resources; Supervision; Writing - original draft; Writing - review & editing.

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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

No data was used for the research described in the article.

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