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Sensitive colorimetric detection of antibiotic resistant *Staphylococcus aureus* on dairy farms using LAMP with pH-responsive polydiacetylene

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ABSTRACT

Rapidly and accurately detecting antibiotic-resistant pathogens in agriculture and husbandry is important since these represent a major threat to public health. While much attention has been dedicated to detecting now-common resistant bacteria, such as methicillin-resistant *Staphylococcus aureus*, fewer methods have been developed to assess resistance against macrolides in *Staphylococcus aureus (SA)*. Here, we report a visual on-site detection system for macrolide resistant *SA* in dairy products. First, metagenomic sequencing in raw milk, cow manure, water and aerosol deposit collected from dairy farms around Tianjin was used to identify the most abundant macrolide resistance gene, which was found to be the *macB* gene. In parallel, *SA* housekeeping genes were screened to allow selective identification of *SA*, which resulted in the selection of the *SAOUHSC_01275* gene. Next, LAMP assays targeting the above-mentioned genes were developed and interpreted by agarose gel electrophoresis. For on-site application, different pH-sensitive colorimetric LAMP indicators were compared, which resulted in selection of polydiacetylene (PDA) as the most sensitive candidate. Additionally, a semi-quantitative detection could be realized by analyzing the RGB information via smartphone with a LOD of 1.344×10^{-7} ng/μL of genomic DNA from a milk sample. Finally, the proposed method was successfully carried out at a real farm within 1 h from sample to result by using freeze-dried reagents and portable devices. This is the first instance in which PDA is used to detect LAMP products, and this generic read-out system can be expanded to other antibiotic resistant genes and bacteria.

1. Introduction

The introduction of antibiotics has significantly reduced morbidity and mortality from infectious diseases worldwide (America, 2011). However, the widespread use of antibiotics has also led to excessive amounts of residues ending up in the environment. Moreover, millions of deaths and financial loss have been caused by antimicrobial resistance (AMR) (Milobedzka et al., 2022). Amongst bacteria with acquired antibiotic resistance, *Staphylococcus aureus* (*SA*) is in the top three in the global ranking (Fair and Tor, 2014). For the treatment of bacterial infections with AMRs, new and effective antibiotics are urgently needed, since antibiotic resistance genes (ARGs) can be spread easily and quickly, but the discovery of new antibiotics is seriously lagging compared to the rate at which bacterial resistance has developed in recent years. As a result, clinically effective antibiotics are being exhausted (Reardon, 2014). For instance, due to limited treatment options with non-beta-lactam antibiotics, bovine mastitis caused by methicillin-resistant *SA* is a major problem in veterinary medicine

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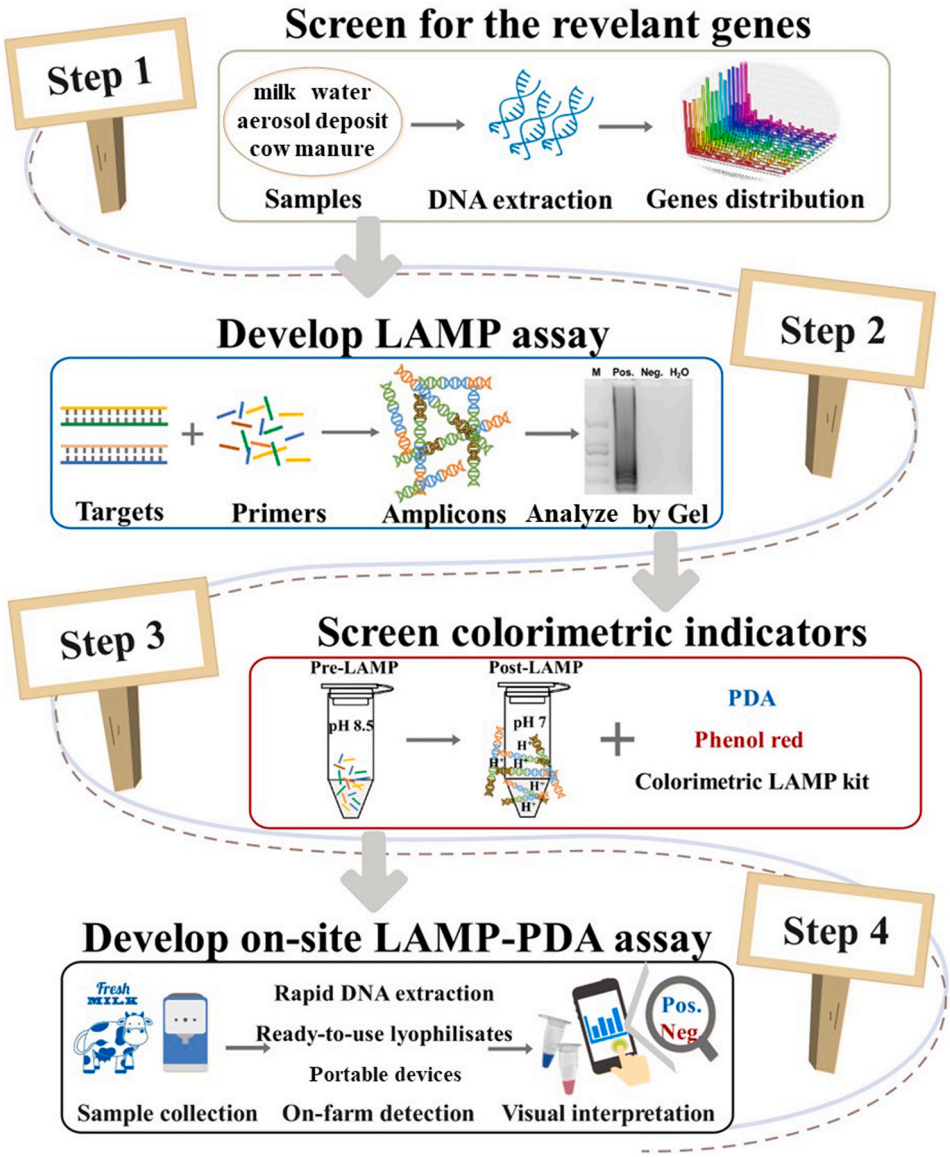

Fig. 1. The workflow for the development of a sensitive, selective and robust on-site DNA detection system for macrolide resistant *SA*.

(Schnitt and Tenhagen, 2020; Shrestha et al., 2021). It is thus crucial to know which bacterium is causing an infection, to determine the most effective use of the currently available antibiotics.

To design adequate treatment strategies, to cure infections, while limiting further spread of AMR, it is crucial to (i) understand the types, abundance, and mechanisms of ARGs, and (ii) develop rapid and reliable methods to detect the presence of ARGs in different bacteria. The morbidity or mortality of the infection depends on the combination of ARGs and specific pathogens, due to their varying susceptibility to different antibiotics (Pal et al., 2016). Multidrug-resistant bacteria can expel different classes of antibiotics through efflux pump systems, including the adenosine triphosphate (ATP)-binding-cassette family (ABC-F) (Crow et al., 2017), in which the *macAB* genes play a vital role (Lu and Zgurskaya, 2013; Wang et al., 2022). However, the detection of these genes has not been extensively studied (Honeycutt et al., 2020). Importantly, these macrolide related ARGs are found on plasmids, transposons, and genomic islands and can therefore be easily transferred horizontally between strains and species (Feβler et al., 2018; Yi et al., 2019). In addition, the *macAB* gene-mediated efflux pump mechanism only expels antibiotics without destroying them, thereby increasing the bacterial exposure to antibiotics, and exacerbating the spread of resistance genes.

In addition to understanding the mechanisms behind resistance, it is crucial to be able to effectively find ARGs-carrying-bacteria to optimize therapy and minimize their spread. At present, the main method for the identification of antibiotic resistance is culture-based, including assessment of morphological/biochemical characteristics of the colonies and antibiotic susceptibility testing (AST). However, these tests are cumbersome, and time-consuming due to the need for bacterial growth, and therefore delays the availability of results (Galhano et al., 2021; Huber et al., 2020; Rodrigues et al., 2020). Alternatively, molecular methods, for example, polymerase chain reaction (PCR)-based assays can be used to identify specific genes (Sanchini, 2022; Voorhuijzen et al., 2020). Although molecular methods can significantly shorten the detection time to 1–3 h, such tests are limited to routine clinical microbiology laboratories, and require specialized equipment and skilled technicians. The latter is restrictive, and the availability of fast and reliable methods with on-site applicability is expected to facilitate early detection of ARGs carrying organisms, as well as provide access to such technology in resource-limited settings (Bhattacharyya et al., 2019).

A well-known candidate for the on-site amplification of genetic material is the loop-mediated isothermal amplification (LAMP) method.

LAMP is based on the use of specially designed inner and outer primers for six regions on the target gene, typically resulting in 10^9–10^{10} folds amplification within 30–60 min under isothermal conditions (Notomi et al., 2000). Recently, LAMP has been proven to be suitable for rapid detection with high sensitivity and specificity (Aartse et al., 2017; Lee et al., 2020; Liu et al., 2020; Roy et al., 2017; Zhang et al., 2019). Table S1 shows a comparison of different on-site detection systems based on LAMP assays, demonstrating that especially colorimetric LAMP assays represent a simple and cost-efficient approach to on-site detection. Specifically, extensive research has been devoted to the use of different pH-indicators to analyze LAMP products (Jaroenram et al., 2019; Roy et al., 2017). Despite their simplicity, however, these assays often suffer from limited sensitivity, and their applicability to real on-site scenario's has not been fully explored. In previous studies, polydiacetylene (PDA), as a class of amphiphilic lipid polymers, has been applied to on-site testing schemes, due to its clear color change (blue--to-red) when it experiences pH changes in solution, which makes it appealing to use in the field of colorimetric biosensors (Camilloto et al., 2021; Li et al., 2020; Nguyen et al., 2020). However, its applicability for detection of LAMP products has not been studied.

The objectives of this study were to 1) screen the main ARGs present in the farms around Tianjin city and housekeeping genes in *SA*; 2) assess PDA as a pH indicator compared to other color indicators for LAMP products; 3) establish a LAMP-PDA system to sensitively and selectively detect macrolide resistant *SA* in milk, manure, water samples and the aerosol deposit on dairy farms; 4) realize rapid detection of macrolide resistant *SA* on the farm.

2. Materials and methods

2.1. Principle and design of LAMP-PDA system for the visual detection of macrolide resistant SA

In this work, we have designed a LAMP-PDA system for the visual detection of macrolide resistant *SA* (Fig. 1). First, bacterial strains and milk from cows suffering from mastitis were collected for metagenomic sequencing (MGS) to obtain genetic information on the bacteria causing the infection. Then, the most abundant resistant gene was selected and a LAMP assay targeting that gene was developed; the detection during this step was done with agarose electrophoresis. However, this assay will detect any sample containing the ARGs in the ecosystem. Moreover, it is essential to identify the bacteria as well as the ARGs, to be truly helpful to give some practical guidelines for prescribing effective antibiotics. Therefore, in parallel, housekeeping genes of *SA* were screened, given that *SA* is one of the most prevalent bacteria in bovine mastitis (Shrestha et al., 2021), and a second LAMP assay, targeting the selected housekeeping gene was developed. In the third step, to ensure robust and sensitive visual interpretation of LAMP products, a comparison was made between colorimetric indicators (PDA, phenol red, and a commercial LAMP kit) for the optimal reporting of the successful amplification by the naked eye or smartphone. Finally, in the fourth step, the conditions and workflow were optimized for on-site detection and applied to testing real milk samples on the dairy farms using freeze-dried LAMP reagents and portable devices to obtain results within 1 h after taking a sample.

2.2. Screening of ARGs and housekeeping genes for SA in real samples

Fresh milk, manure, water and the aerosol deposit from farms in Tianjin, China (Fig. S1) were selected. Then MGS was used to find the most abundant ARG by testing extracted DNA from the samples (see section 1.3 in supporting information, SI). For a highly specific and robust test for one specific type of bacteria, six *SA* housekeeping genes were screened (Table S3), because of their highly conserved regions (Kumar et al., 2021). Several bacterial strains and reference bacterial cultures were used to test the specificity of the housekeeping genes (see section 1.3 and Table S2 in SI), which was done by PCR (see protocol in Table S4).

2.3. Primer design and LAMP assay

The LAMP primers for the most abundant ARGs and housekeeping genes of *SA* were designed by NEB® LAMP primer design tool (https://lamp.neb.com/#!/). Six primers were selected to specifically recognize the distinct regions of the target gene, including a set of two inner primers FIP (F1c + F2) and BIP (B1c + B2), two outer primers (F3 and B3), and two loop primers (LB, LF to accelerate the reaction). The LAMP reaction was carried out in a 25 μL reaction mixture containing 1.25 μL of 10 × isothermal amplification buffer, 3.5 μL 10 mM dNTP Mix, 1.5 μL 100 mM $MgSO_4$, 2.5 μL 10 × LAMP Primers Mix, 1 μL Bst 2.0 DNA polymerase and 2 μL of the template, made up to a final volume of 25 μL with distilled water. The LAMP reaction took place at 64 °C for 40 min. Finally, the products of LAMP were analyzed by running 1.5% agarose gel electrophoresis and visualized by the Amersham Imager.

2.4. Comparison of colorimetric detections systems for LAMP assay

The LAMP assay for the selected ARG was developed by analyzing the LAMP products by Gel, which is not suitable for the on-site detection. Therefore, different colorimetric LAMP indicators (PDA, phenol red and a commercial LAMP visualization kit) were compared to determine which can detect successful amplification most sensitively via induced pH change, without altering the amplification process itself. First, PDA was produced according to previously reported procedures (Li et al., 2020; Li et al., 2022; see section 1.4 in SI). Then, the ARG LAMP assay was carried out by using a monoclonal colony from a cow suffering from mastitis as the positive target, which was confirmed by MGS; wild type *SA* was used as negative control, and sterile water (H_2O) as blank control. After the LAMP reaction, 10 μL of LAMP amplicons was analyzed by 1.5% agarose gel. Next to that, 15 μL of PDA suspension was added to LAMP amplicons. In parallel, different concentrations (50 and 100 μM) of phenol red were added to the LAMP amplicons. The commercial LAMP visualization kit was used according to the manufacturer's instructions. Afterwards, the color of the post-LAMP reaction mixture with each colorimetric candidate solution was photographed within 2 min. Then, the color of each colorimetric detection systems was analyzed by Image J. Moreover, the absorbance of PDA before and after reaction with LAMP solution was analyzed by an UV–Vis spectrophotometer, and the morphology of PDA in the LAMP-PDA system was characterized by SEM.

Then, it was assessed whether the presence of PDA would influence the electrophoresis, by running 1.5% agarose gel electrophoresis of post-LAMP reaction mixture with and without PDA. Finally, the shelf life of the PDA suspension (with optimized concentration) was checked by storing a PDA suspension at 4 °C for 6 months, and then performing the LAMP-PDA analysis with PDA that was stored for different durations. The R/(R + B + G) value was recorded every month.

2.5. Performance of colorimetric LAMP system for detection of the ARG

First, one milk sample, which was confirmed to contain the selected macrolide resistance gene by MGS, was chosen for genomic DNA extraction. The extracted DNA was analyzed by the developed LAMP assay, after which the amplification was checked by both 1.5% agarose gel electrophoresis and colorimetric read-out. In parallel, the conventional PCR assay was also conducted as benchmark. Moreover, semi-quantitative information was obtained by collecting the RGB information of the colorimetric detection system by using the "Color grab" smartphone application and the relationship between the diluted targets and the value of R/(R + G + B) was used to investigate the sensitivity of our developed method. The limit of detection (LOD) was calculated as LOD = 3 SD/b, where SD represents the standard deviation of the blank

samples, and b represents the slope of the calibration curve. The visual LOD was defined as the lowest target concentration to produce a purple color. Finally, the LOD was also confirmed by analyzing the results from gel; the assay was performed 10 times by adding the templates with the concentration of the estimated detection limit and a ten-fold dilution.

Finally, the accuracy of the LAMP-PDA system was investigated with PDA from within and between two batches. Three different concentrations of ARG were analyzed in triplicate to establish the intra-batch precision using the PDA from the same batch, by calculating the relative standard deviation (RSD). Otherwise, for the inter-batch precision with each concentration of ARG, precision was established with 2 different batches of PDA.

2.6. Detection of macrolide resistant SA in the laboratory and on-farm

To identify the macrolide-resistant *SA* strains, and to verify the feasibility of our proposed LAMP assay for the detection of actual samples, 3 fresh milk (Cmi01\02\03) and 2 manure samples (Csh01\02) were obtained from dairy farms in Tianjin and were stored at 4 °C before use. DNA was extracted by boiling in 1% Triton X-100 for 10 min (Sowmya et al., 2012) and MGS was used to assess the distribution of ARGs. The samples were enriched by using 8 μg/mL erythromycin plates. If the minimum inhibitory concentration of *SA* to erythromycin ≥8 μg/mL, then it is considered to be resistant according to the standard established by the Clinical and Laboratory Standards Institute (CLSI-M100-ED31). Then, 30 colonies from different plates were chosen randomly and tested by the developed LAMP, PCR and Sanger sequencing assays.

For on-site detection, the rapid DNA extraction of milk samples was done on-farm; ready-to-use lyophilisates of LAMP reagents were preloaded in PCR tubes, including dNTPs, and LAMP primers, reaction buffer, and Bst. 2.0 enzyme. Meanwhile, a simple and portable shaker, heater, and centrifuge were used. Then the detection was performed by the proposed LAMP assay. More experimental details can be found in Fig. 5 and a video in SI 3.

3. Results and discussion

3.1. Screening of ARGs and housekeeping gene for SA in real samples

To identify which ARG are most prevalent, 15 samples, including fresh milk, manure, river water and the aerosol deposit near the farm were collected and analyzed by MGS. The results (Fig. S2) show the abundance of ARGs in our sample pool, with the *macB* gene being the most abundant. Then 5 samples were also tested by the PCR assay, which confirms that they contain the *macB* gene (Fig. S3). One of these samples was used as the positive sample in subsequent analyses. In parallel, 6 *SA* housekeeping genes were screened by PCR to develop a LAMP assay that is specific for *SA*, which can be carried out together with the assay for the ARG. Two out of the six tested genes showed specificity towards *SA*, namely the *SAOUHSC_01275* and *gmk* genes (Fig. S4).

3.2. LAMP assays for the detection of macB gene and housekeeping genes of SA

The design of LAMP primers is a crucial step in the assay development, as it determines the ultimate assay specificity. Here, a conserved region of the target genes was chosen to design the LAMP primers. The locations and sequences of the primers in *macB* gene and *SAOUHSC_01275* gene are shown in Fig. S5A; for the *gmk* gene, see Table S5 in the SI. After the design of the primer sets, a LAMP assay for *macB* gene detection was established and the amplicons were detected by gel electrophoresis. First, several key parameters were optimized (Fig. S6). Then five milk samples (positive for *macB gene,* confirmed by MGS) were positively analyzed, while no ladder pattern could be observed for the wild type *SA* 8325 (abbreviated *SA* 8325 below) and

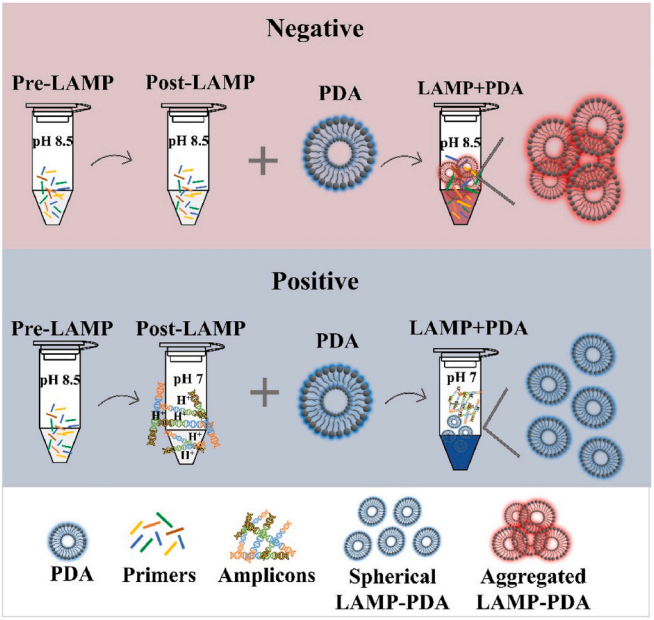

Fig. 2. Schematic of negative and positive LAMP reactions with PDA-based detection.

sterilized water (H_2O) (Fig. S5B), which indicates that this primer set can be used to distinguish the antibiotic resistant bacteria from the non-resistant bacteria. Then, a LAMP assay was developed for both *SAOUHSC_01275* and *gmk SA* housekeeping genes. The assays were tested against a panel of different bacteria. *SAOUHSC_01275* (Fig. S7A) indeed showed high specificity, whereas two false positive results were obtained for the *gmk* gene (Fig. S7B), which is why the LAMP-*SAOUHSC_01275* gene was used in further experiments as the *SA*-specific LAMP assay.

3.3. Feasibility of PDA for visual detection in LAMP assay

Given that the pH of the LAMP system will decrease due to the generation of protons during amplification (Duarte-Guevara et al., 2014), research has increasingly focused on the use of pH indicators, among which phenol red is the most commonly used for the on-site detection (Jun Ji et al., 2020). However, the color change caused by phenol red as an indicator is limited to warm colors (yellow-red), and even when mixed with other indicators to enhance color discrimination, still it is challenging to identify successful amplification of targets at lower concentrations with the naked eye (Scott et al., 2020). To minimize errors in distinguishing colors, an ideal pH indicator should be very sensitive to pH change and have an obvious color distinction within the right pH range. Driven by the pH-responsive behavior of PDA in previous work, we synthesized and optimized a PDA color indicator (Section 1.4 in SI and Fig. S8) as detection strategy for LAMP.

Before the LAMP reaction (pre-LAMP), the pH of the system is ~8.5 for both positive and negative samples. After the LAMP reaction (post-LAMP), a large amount of DNA should be present in positive samples, which is accompanied by the production of protons that lowers the solution pH. In negative samples, however, the LAMP system should remain at the same pH as pre-LAMP. Therefore, theoretically, when the PDA suspension (blue color) is added, the positive LAMP + PDA system should remain blue (lowered pH) and the negative LAMP + PDA should be red (~pH 8.5). This detection scheme is depicted in Fig. 2.

Then, PDA, phenol red and a commercial LAMP detection reagent mixture were tested to visualize the amplification. Unfortunately, no color change was observed for phenol red and commercial LAMP kit, which might be because the LAMP reaction mixture contains buffer,

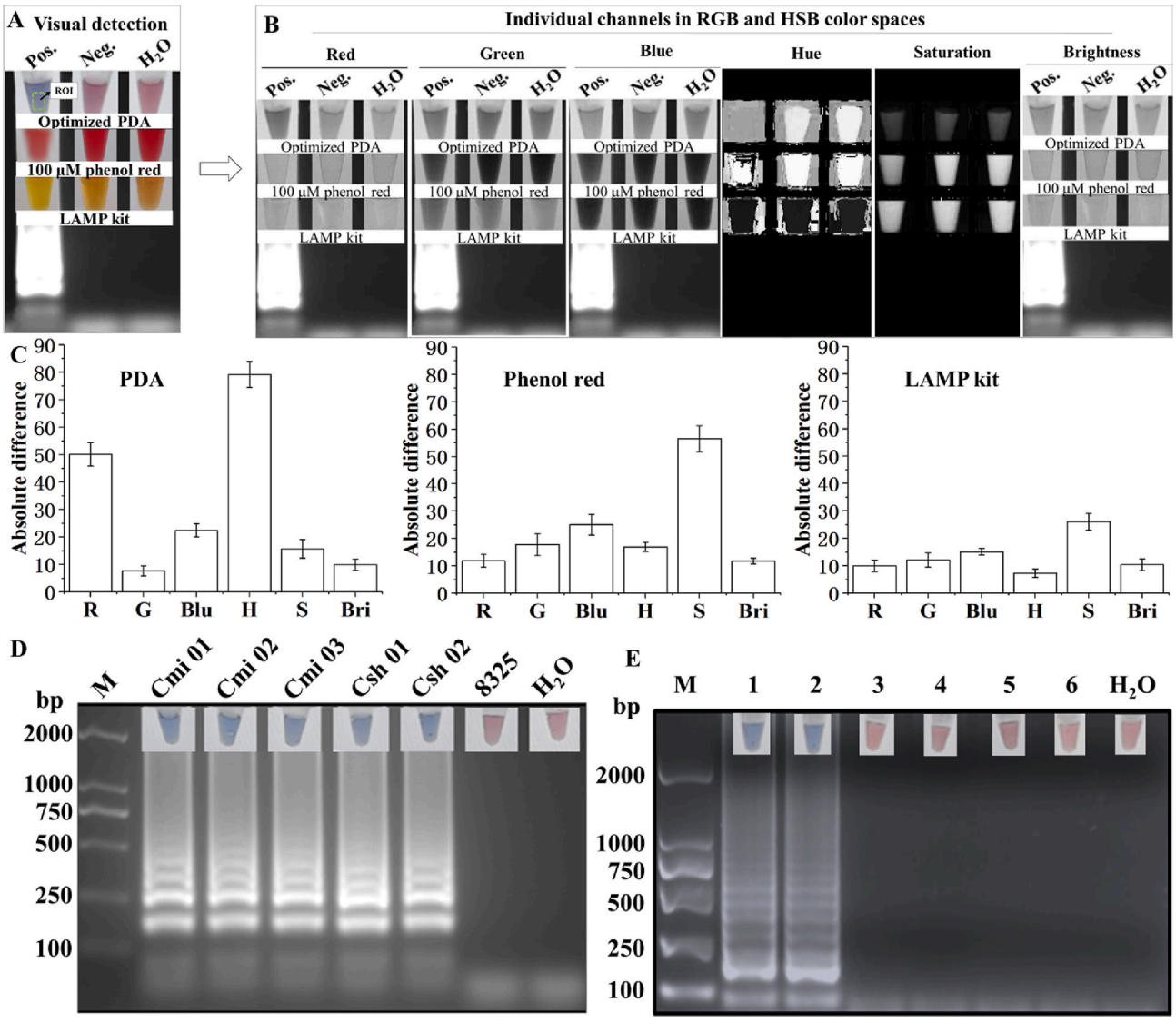

Fig. 3. (A) Agarose gel electrophoresis and visual detection of LAMP products based on different indicators under optimized conditions. Pos: milk sample with *macB* gene-positive; Neg: *SA* 8325 as negative control and sterilized water (H_2O) without any DNA template as blank control. (B) Colorimetric image analysis of individual channels in RGB and HSB color spaces, obtained from analyzing a central region of interest (ROI) from the photograph of the test tube. (C) Difference of each color component between a positive and negative reaction by analyzing the ROI via Image J. Absolute difference (Y value) refers to the absolute value of positive samples minus control samples. Error bars represent the RSD by calculating the color intensity from the colorimetric images three different times. (D) Agarose gel electrophoresis results and color readout of the detection of *macB* gene based on the developed LAMP-PDA assay. Lane M: 2000 DNA ladder, lanes S1–S5: 5 positive monoclonal genes from milk samples, lane 6: *SA* 8325 as negative control, lane 7: sterile water (H_2O). (E) Agarose gel electrophoresis results and color readout of the detection of *SAOUHSC_01275* gene based on the *SA*-specific LAMP-PDA assay. Lane M: 2000 DNA ladder, lanes 1–6: *SA* 8325, *SA* 8325 derivative, *E. coli, Salmomella, Shiga bacillus, Listeria.*

which reduces the magnitude of the pH change, thereby hampering this type of colorimetric reaction. Interestingly though, even in these samples with limited response from phenol red and the commercial kit, when the PDA was added, the results show a color difference between positive (blue/purple) and negative (red) LAMP reactions, though it is not as obvious as the different between pH 7 and pH 8.5 solutions.

3.4. Investigation and optimization of PDA for visual detection in LAMP assay

After having established that the color response of PDA was greater than that of the other dyes, further experiments were performed to explore the color response mechanism (see more details in Table S6, Figs. S9 and S10) and the reaction system was optimized to make PDA exhibit highly sensitive color discrimination.

To further verify that PDA responds more sensitively to a successful LAMP reaction than the other colorimetric reagents, the commercial kit and phenol red were used again to evaluate the results after the reported optimization (Fig. 3A). The color of both the positive and negative LAMP systems after adding phenol red or commercial LAMP kit showed warm colors at both pH values (yellow, pink, red), which makes it difficult to distinguish the positive result from a negative result. On the other hand, the LAMP-PDA system gave a cool color (blue, positive) or a warm color (red, negative), making it easier to differentiate. Next, images of the three colorimetric indicators after LAMP reaction were digitally analyzed (Fig. 3B) in different color spaces (RGB and HSB). When looking at the difference between the positive and negative results it is clear that PDA shows the most sensitivity, especially in the Red and Hue channels (Fig. 3C). Further analysis with respect to concentration dependency revealed the highest R^2 value for R/(R + B + G) (Fig. S11),

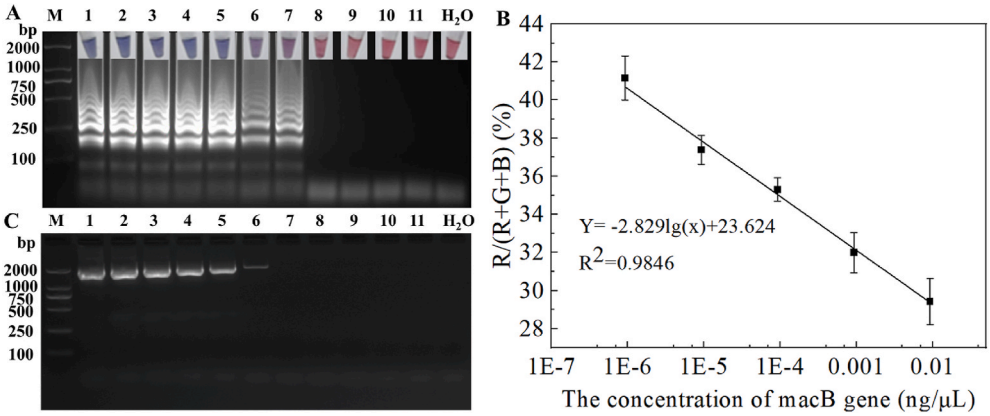

Fig. 4. Detection of genomic DNA from a positive sample based on the developed LAMP-PDA assay. (A) Agarose gel electrophoresis and color of the LAMP-PDA system. (B) Linear correlation between colorimetric signal obtained with the LAMP-PDA system for detecting the *macB* gene, against the concentration of extracted DNA. The error bars represented the standard deviation of three different amplifications; (C) Agarose gel electrophoresis of the PCR assay. In Fig. 4A and C, lane M: 2000 DNA ladder, lanes 1–11: *macB* gene-positive milk sample with the 10-fold serial dilutions of the genomic DNA from 9.248 ng/μL to 9.248 × 10^{-10} ng/μL; lane 12: H_2O.

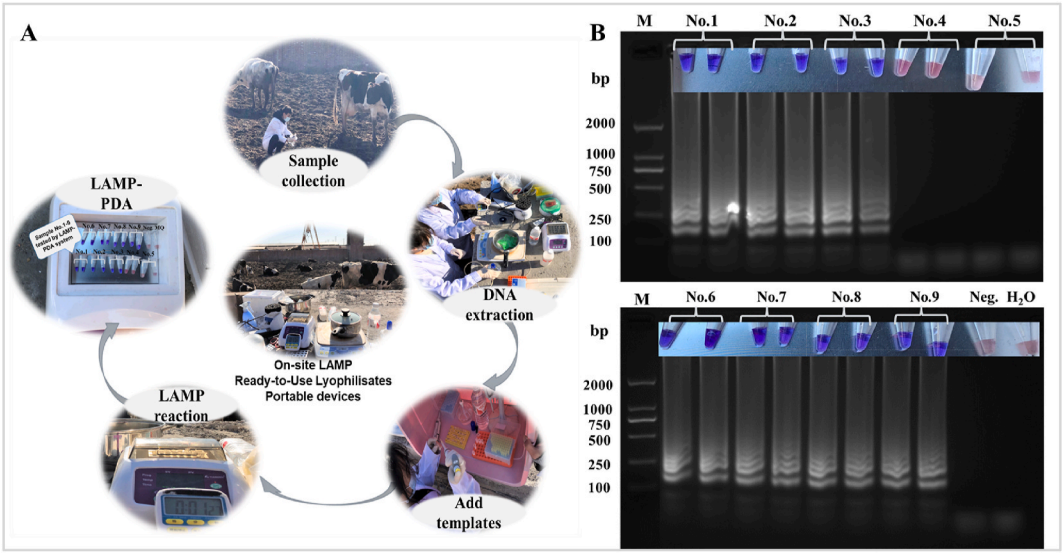

Fig. 5. On-farm detection of *macB* gene based on LAMP-PDA system. (A) Workflow for the on-site detection; (B) Comparison of the results between visual on-site detection and gel electrophoresis.

which was used subsequently as metric. Interestingly, these color channels indicate a color change, which is also visibly interpretable. While it is true that phenol red also showed a clear difference in the saturation channel, this type of change is more difficult to assess by naked eyes.

Finally, the optimized PDA-based detection was used in the LAMP assay for the *macB* gene. All the positive samples showed a blue color, while the *SA* 8325 and blank control showed a red color (Fig. 3D). Additionally, the LAMP-PDA system was used for the *SA* detection, and equally successful; only when *SA* DNA was used, and a ladder-shaped band appeared on gel, did the PDA remain blue after addition to the reaction mixture (Fig. 3E). Given that the color difference of the LAMP-PDA system relies on the drop in pH during LAMP in general, combined with the fact that the PDA suspension can be stably stored for at least 3 months at 4 °C (Fig. S12), it is expected that PDA can be used as a universal indicator for LAMP reactions.

Table 1
The inter batch and intra batch precision of LAMP-PDA system (n = 3).

Concentration (ng/μL)	Inter batch			Intra batch		
	$\overline{X}$*	SD	*RSD* (%)	$\overline{X}$*	SD	*RSD* (%)
9.248 × 10^{-7}	41.5	2.9	6.9	41.9	1.6	3.8
9.248 × 10^{-5}	33.7	1.7	5.1	34.3	0.6	1.9
9.248 × 10^{-3}	29.5	1.8	6.2	29.7	0.9	3.0

Note: $\overline{X}$* represents the average value of R/(R + G + B) × 100%.

3.5. Performance of colorimetric LAMP system for detection of the ARG

Genomic DNA of the milk from cows suffering from mastitis was extracted, and the concentration was determined to be 115.6 ng/μL with good purity and quality (A260/A280 = 1.82, and A260/A230 = 2.09) (Usman et al., 2014). When analyzed by LAMP followed by gel electrophoresis (Fig. 4A) ladder-like bands were obtained for concentrations between 9.248 × 10^{-6} ng/μL and 9.248 ng/μL, which were accompanied by a blue/purple color after adding the PDA suspension. The concentrations below 9.248 × 10^{-6} ng/μL and the blank control showed no amplification products and red color after PDA addition. The estimated LOD was confirmed to be 9.248 × 10^{-6} ng/μL by analysis of 10 samples at this concentration and 10 samples at a 10 × lower concentration (Fig. S13). The visual LOD was defined as 9.248 × 10^{-6} ng/μL (Lane 7), in which a purple color was obtained by addition of PDA. This was investigated by asking 30 people for color discrimination, and 28/30 identified the color of lane 7 as purple (positive), and Lane 8 as red (negative) (Table S7). Considering that some people are less sensitive to color, a smartphone App "Color grab" was used to collect the RGB information and a semi-quantitative detection was realized, in which the

Table 2
Method comparison of PCR and LAMP.

Target	Method	N	TP	TN	FP	FN	specificity (%)	sensitivity (%)	accuracy (%)
SAOUHSC_01275	PCR	30	20	6	0	4	100	83	87
	LAMP	30	23	6	0	1	100	96	97
macB	PCR	30	22	6	0	2	100	92	93
	LAMP	30	24	5	1	0	83	100	97

N is the total number of samples. TP = true positive, TN = true negative, FP = false positive, FN = false negative.

R/(R + G + B) value of the LAMP-PDA suspension showed a linear relationship with the logarithm of the concentration of genomic DNA between 9.248×10^{-7} ng/μL and 9.248×10^{-3} ng/μL, with an estimated LOD of 1.344×10^{-7} ng/μL (Fig. 4B). Meanwhile, the detection limit of LAMP-PDA system was at least 10-fold better than that of the PCR assay (Fig. 4C). Next, the precision of the LAMP-PDA system was evaluated (Table 1). The results show that the RSD with one PDA batch was 1.9%–3.8%, and from two batches below 6.9%. Overall, this proposed method can achieve direct visual detection, as well as smartphone-assisted semi-quantitative detection, with good detection limits and acceptable precision.

3.6. Detection of macrolide resistant SA in the laboratory and on-farm

Another five milk samples were taken at farms, and these were investigated in different ways. First, MGS confirmed that the *macB* gene was the most abundant ARG, and the *SA* was one of the main pathogens found in these cows suffering from mastitis (Fig. S14). Next, these samples were cultured and enriched, to obtain colonies associated with macrolide resistance (Fig. S15). Then, 30 colonies were randomly selected (6 colonies per sample) and tested by the LAMP, and PCR assays, while Sanger sequencing was used to identify true positives (see Figs. S15–S18 and Table 2). Compared to the PCR method, the LAMP assay showed higher sensitivity (100% vs. 92%), and accuracy (97% vs. 93%), but resulted in one false positive for the *macB* gene test (specificity 83% vs. 100%). For the *SAOUHSC_01275* gene test, compared to PCR, LAMP also showed better performance: 96% vs. 83% sensitivity, 100% vs. 100% specificity, and 97% vs. 87% accuracy.

Next, a transportable setup was taken to a farm. Nine milk samples were selected on-farm, and after the rapid DNA extraction, they were tested by the LAMP-PDA system (see workflow in Fig. 5A and a video in SI). As shown in Fig. 5B, the results from the gel and PDA report agreed, which means the LAMP-PDA system can be successfully used as a robust tool for the rapid screening of ARGs. The above results all demonstrate that our method is reliable, sensitive, and user-friendly, and is suitable for in-field and resources limited settings.

4. Conclusion

In conclusion, we have successfully established a sensitive visual LAMP-PDA system to test the macrolide resistant *SA*. The developed LAMP assays for the macrolide resistance gene, *macB* gene and housekeeping gene of *SA* are more sensitive than PCR. Moreover, PDA was used for visual LAMP detection for the first time, with a clear blue (+) or red (−) signal indicating the test result. In addition, to minimize the error of distinguishing colors with the naked eye, semi-quantitative analysis by smartphone was established by collecting the RGB information from the reaction system. Finally, on-farm applicability was demonstrated by detection of macrolide resistant *SA* within 1 h, without any sophisticated laboratory equipment. This PDA-based detecting is a generic strategy, that can be expanded to other antibiotic resistant genes and various bacteria. A current limitation of the method is that it requires the addition of PDA, after the LAMP reaction, similar to using a lateral flow device (LFD) for detection. While this requires opening of the reaction tube, risk of carry-over contamination can likely be preventing by implementing integrated devices, which has also been demonstrated for LFD-based devices (B.H. et al., 2017; Zhang et al., 2020). Moreover, such integration can also further strengthen the approach by allowing tests for multiple targets to be carried out with the same device. Overall, this method has broad application prospects in the field of public health, on-site screening and rapid detection without operational complexity.

CRediT authorship contribution statement

Qiaofeng Li: Conceptualization, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing. **Zhaoxia An:** Methodology, Validation. **Tieqiang Sun:** Data curation, Writing – review & editing. **Shuaifeng Ji:** Methodology, Validation. **Weiya Wang:** Validation. **Yuan Peng:** Formal analysis. **Zhouping Wang:** Resources, Investigation. **Gert IJ. Salentijn:** Conceptualization, Supervision, Writing – review & editing. **Zhixian Gao:** Methodology, Funding acquisition. **Dianpeng Han:** Conceptualization, Supervision, Writing – review & editing.

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

This work is related to our next following research, which is undergoing, and part of the data so far is confidential. If there is a necessity, we will provide it to some extent.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bios.2022.114824.

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Further reading

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