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Detection Sensitivity of Chip Digital PCR for *Staphylococcus aureus*

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Abstract [**Objectives**] This study aimed to evaluate the detection sensitivity of *Staphylococcus aureus* in dairy products utilizing the chip digital PCR (cdPCR) technique. [**Methods**] Specific primers and probes were designed and synthesized based on the conserved sequence of the heat-resistant nuclease gene *nuc* of *S. aureus*. cdPCR was employed to detect *S. aureus*, and the sensitivity of this technique was systematically assessed in samples exhibiting low levels of contamination. [**Results**] cdPCR demonstrated precise quantification when the initial concentration of the sample enrichment solution was equal to or greater than 50 CFU/mL. The detection dynamic range extended across at least five orders of magnitude, with a minimum DNA detection limit of 0.230 4 pg/μL. In artificially contaminated cheese samples, the method's lower limit of quantification for detecting *S. aureus* was 8×10^2 CFU/g. Regression analysis demonstrated that the gene copy number concentration measured by cdPCR exhibited a strong linear correlation with bacterial contamination concentration across a broad range. [**Conclusions**] The cdPCR method developed in this study demonstrates high sensitivity and robust quantitative capabilities, offering a reliable technical approach for the precise detection of low-level *S. aureus* contamination in dairy products.

Key words *Staphylococcus aureus*, Chip digital PCR (cdPCR), Sensitivity

0 Introduction

Staphylococcus aureus is a prevalent foodborne pathogen in the natural environment, known for producing heat-resistant enterotoxins, and constitutes a primary cause of food poisoning[1-2]. The risk of foodborne illness notably escalates when the bacterial concentration exceeds 10^5 CFU/g[3]. Dairy products, particularly raw milk, are highly vulnerable to contamination by *S. aureus*. Contamination from the breeding environment and processing stages constitutes the primary source of contamination in dairy products[4-5]. Food poisoning outbreaks resulting from *S. aureus* contamination are prevalent globally, representing an ongoing public health concern. Consequently, this pathogen has become a significant focus within the food industry[6-7]. Currently, the detection of *S. aureus* primarily depends on traditional national standard methods, which are characterized by lengthy detection time, complex procedures, and an inability to satisfy the demand for rapid results in short shelf-life products[8-9]. Additionally, quantitative PCR (qPCR) is susceptible to inhibition by sample matrices when detecting low concentrations of pathogens and does not provide absolute quantification[10]. Digital PCR (dPCR) is an advanced nucleic acid detection technology that enables absolute quantification without the need for a standard curve. It directly determines the absolute copy number of the target gene in a sample by employing extreme dilution and Poisson distribution analysis. Compared to qPCR, dPCR offers greater accuracy and sensitivity in quantitative detection, as well as enhanced tolerance to inhibitors[11-15]. Therefore, dPCR offers novel approaches and methods for detecting *S. aureus* in dairy products. This study employed chip digital PCR (cdPCR) utilizing nano-microplates to analyze various samples contaminated with *S. aureus*, aiming to evaluate the detection sensitivity of this technique and to provide reference data for the rapid quantitative detection of *S. aureus* in milk and dairy products.

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1 Materials and methods

1.1 Materials

1.1.1 Strains and reagents. The study utilized the standard strain *S. aureus* ATCC 25923. The culture media and reagents employed included blood agar plates, 7.5% sodium chloride broth, Baird-Parker agar medium, magnetic bead-based bacterial DNA extraction kits, PCR probe premix detection solution, and *S. aureus* nucleic acid detection kits.

1.1.2 Instruments and equipment. The instruments and equipment utilized in this study comprised the 1589 biosafety cabinet, SPX-250II biochemical incubator, GR110DA autoclave, EZ2 Connect nucleic acid extractor, QIAcuity One integrated nanochip digital PCR system, Pico17 small centrifuge, nucleic acid protein analyzer, and LightCycler 96 fluorescence quantitative PCR instrument.

1.2 Methods

1.2.1 Synthesis of primers and probes. Primers and probes were designed based on the conserved sequence of the heat-resistant nuclease gene *nuc* of *S. aureus* and were synthesized by Suzhou Genewiz Biotechnology Co., Ltd. The upstream primer sequence was

5'-AGCATCCTAAAAAAGGTGTAGAGA-3', and the downstream primer sequence was 5'-CTTCAATTTTMTTTGCATTTTCTACCA-3'. The fluorescent probe sequence was 5'-HEX-TTTTCGTAAATG-CACTTGCTTCAGGACCA-BHQ1-3'.

1.2.2 Bacterial enrichment method. A pure bacterial solution was prepared by transferring 500 μL of the standard strain glycerol stock into 10 mL of BHI broth, followed by incubation at 36 ℃ for 24 h to allow resuscitation. For the test samples, 25 g (solid) or 25 mL (liquid) of the sample was combined with 225 mL of sterilized 7.5% sodium chloride broth and incubated at 36 ℃ for 18 h prior to subsequent genomic DNA extraction.

1.2.3 Extraction of DNA from *S. aureus*. For the pure bacterial solution, 200 μL of the enriched bacterial solution was combined with 20 μL of lysozyme (50 mg/mL) and incubated at 37 ℃ for 30 min. Subsequently, genomic DNA was automatically extracted using a magnetic bead-based bacterial DNA extraction kit in conjunction with an automated nucleic acid extractor.

For the sample enrichment solution/liquid or solid sample diluent, 5 mL was collected and centrifuged at 4 000 rpm for 10 min. After discarding the supernatant, the precipitate was suspended in sterile saline, washed, and centrifuged; this procedure was repeated twice. Subsequently, 200 μL of enzyme-free sterile water was added to resuspend the sample. Next, 20 μL of lysozyme (50 mg/mL) was added, and the mixture was incubated at 37 ℃ for 30 min. DNA was extracted from the incubated sample using the same procedure as that applied to the pure bacterial solution. The concentration of DNA and the absorbance ratio at 260/280 nm were measured using a nucleic acid protein analyzer to assess the quality of the extracted genomic DNA.

1.2.4 Establishment of cdPCR system. In a 0.2 mL reaction tube, a 40 μL reaction mixture was prepared by combining 10 μL of PCR Mix premix, 2.4 μL each of 10 μmol/L upstream and downstream primers, 0.8 μL of 10 μmol/L probe, 20.4 μL of sterilized deionized water, and 4 μL of template. The test sample template consisted of DNA extracted from the target strain. The positive control template was verified genomic DNA from *S. aureus*. The negative control contained a sample lacking the target DNA. The no template control (NTC) comprised enzyme-free sterile water. The mixed system solution was transferred into a 24-well nano-microplate chip. During sample addition, the solution was introduced along the sidewall to prevent bubble formation. Subsequently, the microplate was covered with a blue sealing film, which was firmly pressed using a roller to ensure proper sealing. The sealed plate was then placed into the instrument for analysis according to the predetermined program. The reaction protocol consisted of an initial pre-denaturation step at 95 ℃ for 2 min, followed by 40 cycles of denaturation at 95 ℃ for 15 sec and annealing at 57 ℃ for 60 sec.

1.2.5 Sensitivity test of *S. aureus* broth enrichment solution. The laboratory-maintained strain *S. aureus* ATCC 25923 was inoculated into BHI broth and incubated for 18 h. Following incubation, the broth enrichment culture was serially diluted to obtain samples with concentrations of 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10^1, and 10^0 CFU/mL for subsequent analysis. Quantification of *S. aureus* was performed using the established cdPCR detection system. Meanwhile, the live bacterial count of the samples was determined using blood agar plates to assess the detection sensitivity of the established method for *S. aureus* broth cultures. Concurrently, fluorescence quantitative PCR was employed for verification purposes.

1.2.6 Sensitivity test of low-level contamination samples of *S. aureus*. 25 g sample of commercially available cheese was inoculated with *S. aureus* bacterial suspension at an inoculation level of 1 – 5 CFU/25 g. Subsequently, 225 mL of sterilized 7.5% sodium chloride broth was added, and the mixture was incubated at 36 ℃ for 18 h. Following incubation, the enrichment solution was serially diluted 10-fold to obtain eight samples corresponding to the original solution and dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}. Quantitative analysis of *S. aureus* was performed using the established cdPCR detection system. Meanwhile, the viable bacterial count of the enrichment solution was determined using Baird-Parker agar plates to assess the detection sensitivity of this method for samples with low-level contamination of *S. aureus*. Concurrently, fluorescence quantitative PCR was employed for verification purposes.

1.2.7 Sensitivity test of genomic DNA copy number of *S. aureus*. DNA was extracted from a pure suspension of *S. aureus*, and its concentration was quantified using a nucleic acid and protein analyzer. Subsequently, the DNA was serially diluted 10-fold with sterile enzyme-free water and used as test templates. The genomic DNA copy number of *S. aureus* was quantitatively assessed by droplet digital PCR (ddPCR) to determine the lower detection limit of this method. Concurrently, quantitative fluorescence PCR was employed for validation purposes.

1.2.8 Quantitative analysis of simulated contaminated cheese samples. Seven artificially contaminated cheese samples were prepared by inoculating them with *S. aureus* suspensions at varying concentrations, specifically 8×10^8, 8×10^7, 8×10^6, 8×10^5, 8×10^4, 8×10^3, and 8×10^2 CFU/10 g of cheese. No enrichment procedures were performed. DNA was extracted from *S. aureus* following direct dilution with normal saline and subsequently subjected to cdPCR analysis to assess the detection sensitivity of the cdPCR method for *S. aureus* in these simulated contamination samples. Concurrently, the results were validated using quantitative fluorescence PCR.

1.2.9 Data processing. The data were analyzed using Microsoft Excel 2016 and Minitab 16 software.

2 Results and analysis

2.1 Sensitivity test of broth enrichment solution DNA was extracted from a 10-fold serial dilution of the enrichment solution containing *S. aureus* cultured in BHI broth for 18 h, for subsequent cdPCR analysis. As shown in Table 1, positive partitions were detectable using this method when the bacterial suspension concentration ranged from 9.0×10^1 to 9.0×10^8 CFU/mL. Notably, samples 1 and 2 exhibited excessively high concentrations, resulting in near saturation of positive fluorescent dots. In contrast, samples 3 – 7 demonstrated clear differentiation between positive and negative microdroplets, indicating that the template concentrations in these samples were optimal. The results of fluorescence quantitative PCR analysis demonstrated that cdPCR was capable of detecting BHI broth enrichment solutions of *S. aureus* at a minimum concentration of 90 CFU/mL, whereas qPCR detected the solution at a minimum concentration of 900 CFU/mL. These findings indicate that cdPCR exhibits higher detection sensitivity, with a detection copy number concentration as low as 0.55 copies/μL.

Table 1 Sensitivity test of broth enrichment solution of *Staphylococcus aureus*

Sample No.	Concentration of bacterial suspension // CFU/mL	cdPCR detection copies/μL	*Ct* value of qPCR detection
1	9.0×10^8	n. a	13.40
2	9.0×10^7	n. a	16.92
3	9.0×10^6	68 869	19.90
4	9.0×10^5	10 402	23.26
5	9.0×10^4	780.1	27.92
6	9.0×10^3	90.14	31.52
7	9.0×10^2	6.44	35.36
8	90	0.55	0
NTC	–	0	–

NOTE n. a indicates saturation of the fluorescent dots, suggesting that the template requires dilution prior to detection; NTC refers to a template-free control. The same below.

2.2 Sensitivity test for samples with low-level contamination Following the enrichment of low-level artificially contaminated cheese samples in 7.5% sodium chloride broth for 18 h, the enrichment solution was subjected to 10-fold serial dilution, and DNA was subsequently extracted for cdPCR analysis. As presented in Table 2, when the bacterial suspension concentration ranged from 5.0×10^1 to 5.0×10^8 CFU/mL, positive partitions were detectable using this method. Furthermore, positive and negative microdroplets in samples 3 – 7 were clearly distinguishable, indicating that the template concentration was appropriate. Based on the results of fluorescence quantitative PCR analysis, it was observed that when the target bacterial concentration in the sample was 50 CFU/mL, no *Ct* value was obtained. This finding indicates that for samples with low levels of contamination, cdPCR detection demonstrates higher sensitivity and can provide accurate quantification. Furthermore, when the initial concentration of the sample enrichment solution was 50 CFU/mL or higher, the method accurately detected the gene copy number of the target bacteria, with a minimum detectable concentration as low as 1.1 copies/μL.

Table 2 Sensitivity test of low-level contaminated samples of *Staphylococcus aureus*

Sample No.	Concentration of bacterial enrichment solution CFU/mL	cdPCR detection copies/μL	*Ct* value of qPCR detection
1	5.0×10^8	n. a	14.40
2	5.0×10^7	n. a	15.97
3	5.0×10^6	62 911	19.75
4	5.0×10^5	9 777	23.63
5	5.0×10^4	733	28.40
6	5.0×10^3	54.90	32.06
7	5.0×10^2	7.44	35.83
8	50	1.10	–
NTC	–	0	–

2.3 Detection sensitivity of genomic DNA copy number The genomic DNA of *S. aureus* was serially diluted 10-fold, and its concentration was measured using a nucleic acid and protein analyzer. The gene copy number of *S. aureus* was quantitatively assessed via cdPCR. As shown in Table 3, cdPCR detection effectively covered at least five concentration gradients, with the lowest detectable DNA concentration being 0.230 4 pg/μL. At this concentration, no *Ct* value was obtained from fluorescence PCR, resulting in a negative detection outcome. Within the DNA concentration range of 0.230 4 to 2.030 4 ng/μL, positive and negative partitions were clearly distinguishable, indicating that cdPCR exhibits robust quantitative capability across a broad spectrum of DNA concentrations. Compared to conventional fluorescence quantitative PCR technique, cdPCR offers an absolute advantage in the quantification of nucleic acids at extremely low concentrations.

Table 3 Sensitivity test of genomic DNA of *Staphylococcus aureus*

Sample No.	DNA template concentration	cdPCR detection copies/μL	*Ct* value of qPCR detection
1	230.4 ng/μL	n. a	14.37
2	23.04 ng/μL	n. a	17.43
3	2.304 ng/μL	5 9457	21.72
4	0.230 4 ng/μL	6 486	26.84
5	23.04 pg/μL	491.20	30.71
6	2.304 pg/μL	36.62	34.82
7	0.230 4 pg/μL	2.26	–
NTC	–	0	–

Seven cheese samples (10 g each) were artificially contaminated with varying concentrations of *S. aureus*. Each sample was diluted with 90 mL of sterile normal saline, followed by direct DNA extraction for cdPCR analysis. The detection results are presented in Table 4, and the corresponding one-dimensional scatter plot is illustrated in Fig. 1. Quantitative detection of *S. aureus* in cheese by cdPCR was achievable without enrichment when the contamination level reached or exceeded 8×10^2 CFU/g. For qPCR, the contamination concentration must be at least 8×10^3 CFU/g to ensure accurate detection of the target strain. Regression analysis of samples 1 – 6 indicated that, after logarithmic transformation, the copy number concentration detected by cdPCR exhibited a strong linear correlation with the contamination concentration of the tested samples, with a coefficient of determination (R^2) of 99.8%. The corresponding fitted regression line is presented in Fig. 2.

Table 4 Experimental results of cdPCR quantitative detection of simulated contaminated cheese samples

Sample No.	Contamination concentration CFU/g	cdPCR detection copies/μL	*Ct* value of qPCR detection
1	8×10^7	72 592	20.12
2	8×10^6	9 006	23.32
3	8×10^5	788.30	26.94
4	8×10^4	98.28	30.95
5	8×10^3	7.74	34.58
6	8×10^2	0.54	–
7	80	0.00	–
Positive control	–	5 548	23.96
Negative control	–	0	–

NOTE The positive control consisted of genomic DNA from *Staphylococcus aureus*, while the negative control was a blank cheese sample.

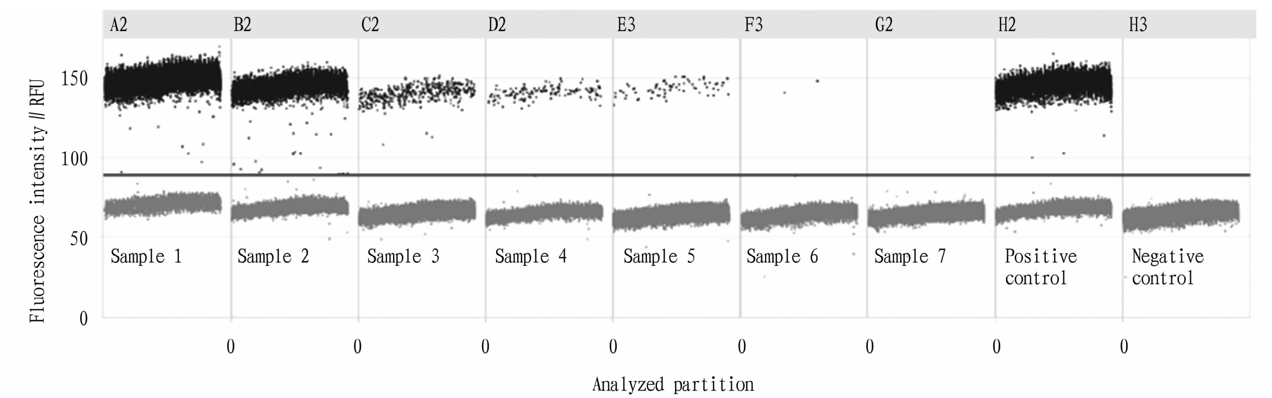

Fig. 1 One-dimensional scatter plot for quantitative detection of simulated contaminated cheese samples

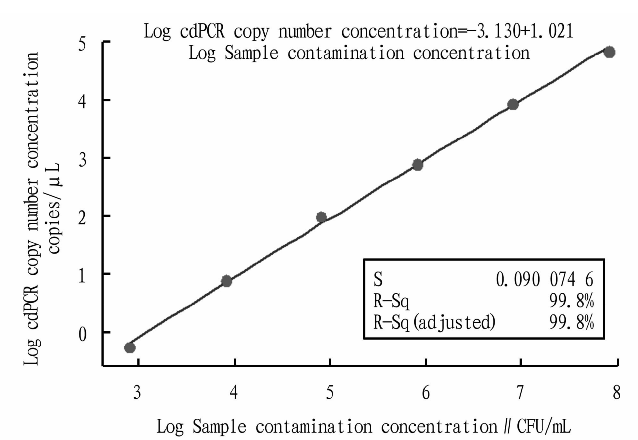

Fig. 2 Fitted regression line of cdPCR copy number concentration and sample contamination concentration

3 Discussion

This study primarily conducted a systematic evaluation of the sensitivity of cdPCR detection method for *S. aureus*. Experimental investigations focused on determining the minimum detectable concentration of *S. aureus* genomic DNA, as well as the lowest detectable concentrations of *S. aureus* in broth culture medium, enrichment medium, and simulated contamination samples of dairy products. The results demonstrated that this method could detect a minimum bacterial concentration of 50 CFU/mL in the sample enrichment solution, and the minimum detectable template DNA concentration was 0.230 4 pg/μL. Using an artificially contaminated cheese sample as an example, the minimum detectable concentration of *S. aureus* in the solid matrix of dairy products by this method was 8×10^2 CFU/g. The detection sensitivity of this method was one to two orders of magnitude greater than that of the qPCR method.

cdPCR was introduced relatively recently and has seen limited application in the field of food testing. In contrast, ddPCR has been successfully employed in detecting pathogenic microorganisms, analyzing source components, identifying allergens, detecting genetically modified components, *etc.* [16-20]. In the detection of foodborne pathogenic bacteria, Zhao Liqing *et al.* [21] employed ddPCR to detect *Salmonella*, achieving a minimum detectable bacterial concentration of 53 CFU/mL. Similarly, Zhou Wei *et al.* [22] utilized dPCR technique to detect *S. aureus* in dairy products, reporting a detection sensitivity of 3.3×10^1 CFU/g. Mao Zhenzhen *et al.* [23] employed multiplex dPCR utilizing cdPCR technique to detect *S. aureus*, *Salmonella*, and *Listeria monocytogenes*. The detectable concentration of the bacterial mixed samples ranged from 10^5 to 10^8 CFU/mL. With ongoing advancements in dPCR technique, cdPCR holds significant potential for application owing to its advantages, including precise quantification, robust resistance to interference, and high sensitivity.

Research has identified multiple factors influencing the sensitivity of dPCR detection[24-27]. These factors include the initial concentration of samples, variations in template DNA extraction methods, the quality of the template DNA, and critically, the number of dispersed reaction systems, specifically the number of effective partitions. The quantitative performance of dPCR is strongly influenced by both the number and uniformity of partitions within its reaction system. Increasing the number of partitions improves the capture efficiency of low-abundance targets, thereby lowering the detection limit. Additionally, these independent partitions provide a basis for multiplex detection. However, variations in partition volume can introduce quantitative errors, representing a critical factor that influences measurement precision and is inversely correlated with detection sensitivity[28-29]. In contrast, cdPCR employs industrially standardized chips and physically separates reaction units using barriers, thereby ensuring a high level of consistency in reaction volume control. This intrinsic structural feature theoretically confers superior measurement stability and accuracy relative to ddPCR[30]. This study utilized an innovative nanochip. Following the addition of the reaction mixture to the chip, it was inserted into the QIAcuity system. The instrument automatically introduced the sample into the nanoholes of the microfluidic chip and independently sealed each hole. By integrating micropartitioning, thermal cycling, and imaging into a unified dPCR system, detection efficiency was significantly enhanced, offering a novel technical approach for the analysis of samples with low levels of contamination.

4 Conclusions

This study employed artificially contaminated samples at varying concentrations to investigate the detection sensitivity of the cdPCR method for *S. aureus* in samples with low levels of contamination. When the initial concentration of the enrichment solution for artificially contaminated cheese samples reached 50 CFU/mL or higher, the gene copy number concentration of *S. aureus* could be accurately quantified. cdPCR detection of *S. aureus* demonstrated a dynamic range spanning at least five orders of magnitude, with a minimum detectable DNA concentration of 0.230 4 pg/μL. The lowest contamination level of *S. aureus* in cheese samples quantitatively identified by cdPCR was 8×10^2 CFU/g. Regression analysis revealed that the copy number concentration measured by cdPCR exhibited a strong linear correlation with the sample contamination concentration within a defined range. The cdPCR detection method for *S. aureus* demonstrates high sensitivity, thereby offering reliable data to support the quantitative detection of low-level contamination in dairy products.

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erated significant revenue from technology transfers, and driven an increase of over 3 000 yuan per hectare in related industries. This truly embodies the principle of writing theses on the land and applying outcomes in the industry.

4.2 Improving the dual-pronged assessment and evaluation system It is necessary to establish an integrated "Party Building + Research" assessment mechanism that incorporates Party members' contributions in areas such as tackling technical challenges, transforming research outcomes, and serving agriculture, rural areas, and farmers into the evaluation of Party building work. Simultaneously, the level of participation in Party building activities and political performance are taken as important references in research performance evaluation and professional title promotion, thereby achieving mutual articulation and reinforcement between the two assessment systems.

4.3 Deepening the collaborative mechanism of joint Party building "Upward Joint Building" involves forming alliances with universities and research institutes to conduct joint research, focusing on basic research areas such as germplasm resource innovation. "Downward Joint Building" involves forming alliances with local governments and agricultural cooperatives to establish experimental demonstration bases, accelerating the application of technologies. "Horizontal Joint Building" involves forming alliances with enterprises to connect the entire "breeding-propagation-promotion" industry chain, enabling rapid translation of research outcomes into tangible income growth for fruit growers.

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