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A recent technique for the detection of *Bacillus cereus* in milk

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Abstract

The occurrence of *Bacillus cereus* in milk and dairy products is attributed to deficient hygiene protocols, consequently resulting in two gastrointestinal illnesses: rare emetic sickness and diarrheal illness triggered by enterotoxins. The current study aimed to overcome the limitations of PCR in detecting low bacterial concentrations through the incorporation of unmodified gold nanoparticles (GNPs) in polymerase Chain Reaction (PCR). *Bacillus cereus* in milk was analyzed using the GNPs-assisted PCR method to detect the *nhe*A gene in comparison to conventional PCR and SYBRGreen qPCR. The *nhe*A gene is a key virulence factor that encodes a protein involved in the generation of non-hemolytic enterotoxins. The result showed adding GNPs to PCR reactions boosted DNA yields and enabled detection down to 10^2 copies of *Bacillus cereus* DNA, versus 10^3 with standard PCR. GNPs-assisted PCR detected *Bacillus cereus* for spiked milk samples at 10^2cfu/ml, while conventional PCR required 10^3cfu/ml. SYBRGreen qPCR also achieved detection at 10^2 DNA copies and 10^2cfu/ml for spiked milk. GNPs-assisted PCR specifically amplified *Bacillus cereus*, not other bacteria like *Bacillus subtilis*, *E. coli*, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Salmonella*, demonstrating assay specificity. Overall, GNPs improved PCR sensitivity for *Bacillus cereus* detection.

Keywords: - Enterotoxigenic; *Bacillus cereus*; milk; PCR; Gold nanoparticles; SYBRGreen.

1. Introduction

In the realm of food safety and quality, milk and dairy products hold significant importance due to their nutritional value and widespread consumption by humans **[1]**. However, the presence of harmful pathogens in these products can pose a significant risk to consumers. One such pathogen that requires attention is *Bacillus cereus*, a Gram-positive spore-forming bacteria found throughout the environment **[2]**. It can contaminate milk and dairy products at any point during the production, processing, and storage process **[3]**. Once in milk, it can survive pasteurization, making it a significant concern for processed milk products **[4]**. It can cause spoilage of milk and dairy products by producing enzymes proteases, lecithinase, lipases, and phospholipases **[5]**. Furthermore, *Bacillus cereus* can cause rare emetic sickness by heat-stable cereulide toxin(*ces*) and diarrheal illnesses in humans triggered by heat-labile enterotoxins such as hemolysins (*hbl*), nonhemolytic enterotoxins (*nhe*), cytotoxins(*cyt*K) leading to food poisoning **[6]**.

Historically, the customary techniques for identifying *Bacillus cereus* have encompassed culture-dependent protocols, quantification of viable cells, microscopic analysis, biochemical assays **[7-9]**, and ELISA **[10]**. However, these approaches harbor certain limitations such as time-consuming and inadequate sensitivity and specificity for discerning low concentrations of *Bacillus cereus* **[11,12]**. To address these challenges, scientists have undertaken innovative approaches, exploring the realms of molecular biology and nanotechnology to improve the detection and quantification of *Bacillus cereus* spores in dairy products **[13,14]**. One such molecular technique that has shown promising results is the PCR, which employs specific primers targeting genes associated with *Bacillus cereus*' virulence, such as (*nhe*, *hbl*, *cyt*K, and *ces*) toxins. This method enables rapid and highly sensitive detection of *Bacillus cereus* in milk and dairy products **[15]**. Notably, several studies have

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consistently revealed a higher detection rate of the *nhe*ABC gene by PCR compared to the *hbl*CDA, *cyt*K, and *ent*FM enterotoxin genes across all isolates. Some studies have even omitted reporting on the *ces* gene **[15-18]**. Of particular significance, the *nhe*A gene serves as a pivotal virulence factor, encoding a protein crucial for the production of non-hemolytic enterotoxins **[19]**. The PCR-based detection of *Bacillus cereus* can be performed in diverse formats, including conventional PCR **[4,15,20]**, Real-time PCR with the use of fluorescent probes **[11,21,22]**, and Real-time PCR SYBRGreen method **[23-25]**. However, it is essential to acknowledge that PCR does have certain limitations. False-negative results may occur due to DNA degradation or the presence of inhibitory substances in food matrices, and the cost of probes can be relatively high **[26,27]**. Therefore, complementary methods have been developed to improve the detection of *Bacillus cereus* by PCR. One of these methods is the use of gold nanoparticles (AuNPs) in combination with conventional PCR as enhancers promising results in terms of sensitivity and specificity **[14,28]**.

GNPs have been introduced as a new supplement in PCR to prevent nonspecific binding even at reduced annealing temperatures and to augment the overall reaction. Two explanations exist that may clarify the mechanism behind the improved reaction efficiency seen with the addition of GNPs: one hypothesis suggests amplification is increased due to surface interactions between the GNPs and PCR reagents, while the other proposes the enhancement stems from the high thermal conductivity and heat capacity of GNPs enabling more efficient thermal transfer **[29,30]**. In sight of these facts, the current study aimed to incorporate the unmodified GNPs assisted PCR to overcome the limitations of PCR for the detection of low enterotoxigenic *Bacillus cereus* concentrations in milk. *Bacillus cereus* contamination was analyzed using the GNPs-assisted PCR method to detect the *nhe*A gene in comparison to conventional PCR and SYBRGreen qPCR.

2. Materials and Methods

2.1. Materials

Luria Bertani (LB) medium, nutrient agar (HiMedia, India). *Bacillus cereus* (ATCC 11778), *Bacillus subtilis* (ATCC 6633), *E. coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 6538), *Listeria monocytogenes* (ATCC 35125), and *Salmonella enteritidis* (MW 362308) were obtained from Food Hygiene Department, Animal Health Research Institute, Egypt. Power Prep DNA Extraction Kit (E0002) from Food and Feed (kogene, Korea). *nhe*A oligonucleotide primers (Willowfort, UK). DNA polymerase cosmo red PCR master mix (W1020300X) and HERA PLUS SYBRGreen (WF1030800X) qPCR master mix (Willowfort, UK). Agarose, Tris base, boric acid, and EDTA (Sigma Aldrich, USA). Red safe cat No 21141 (Intron bio, USA). DNA marker (GeneDirex, USA). hydrogen tetrachloroaurate trihydrate (HAuCl4) and trisodium citrate dihydrate (Alfa Aesar, UK).

2.2. Bacterial culture preparation

All bacterial strains were grown on LB broth overnight at 37℃ in a rotary shaker. The viable cells for each bacterium were determined by surface plating 0.1 ml of the LB broth on to nutrient agar at 37℃ for 24hrs.

2.3. Detection of enterotoxigenic *Bacillus cereus* in spiked milk

Pasteurized milk samples were purchased from the local supermarkets and determined to be negative for enterotoxigenic *Bacillus cereus* by standard culture methods. Different concentrations of enterotoxigenic *Bacillus cereus* solution were spiked into milk samples at final concentrations ranging from 10^7cfu/ml to 10^1cfu/ml. The suspension containing enterotoxigenic *Bacillus cereus* was analyzed using conventional PCR, GNPs-assisted PCR, and Real-time SYBRGreen PCR method. The control samples were not spiked with enterotoxigenic *Bacillus cereus*. All tests were carried out in triplicate.

2.4. Preparation of DNA template

Genomic DNA from bacterial cultures was harvested by boiling method according to (**Zhang et al.,2014**) **[31]**. Genomic DNA from spiked milk samples was harvested by Power Prep DNA Extraction Kit from Food and Feed (E0002) according to the manufacturer manual.

2.5. Establishment of conventional PCR and SYBRGreen(qPCR) for enterotoxigenic *Bacillus cereus* detection

To amplify the 755 bp and 117 bp species-specific fragments for enterotoxigenic *Bacillus cereus*, two pairs of oligonucleotide primers specific for the *nhe*A gene **(Table 1)** were used.

2.6. Synthesis and characterization of GNPs

In accordance with Zabetakis et al.'s study in 2012 **[34]**. Briefly, 50 ml of 0.02% HAuCl4 aqueous solution was heated to boiling with rapid stirring. Then, 1.176 ml of 5% sodium citrate was introduced into the solution under reduced stirring speed. This resulted in a distinct color transition, progressing from pale yellow to shades of grey, black, and deep violet, before finally settling into a ruby red hue. The solution was subsequently cooled to room temperature.

Comprehensive characterization of the synthesized GNPs was carried out utilizing several analytical techniques. Transmission electron microscopy (TEM) using a JEOL-JEM-2100 instrument allowed visualization of individual particle size, shape, and agglomeration state. Dynamic light scattering (DLS) provided data on average particle size and polydispersity index (PDI). Additionally, UV-vis absorbance spectroscopy was used to analyze the optical properties of the GNPs.

2.7. Conventional PCR

PCR amplification was carried out according to Guinebretière et al. **[32]** in 15µl reaction volumes containing 7.5µl cosmo red PCR master mix, 1µl of a 10 pmol primer mix, 1µl template DNA, with the balance made up by PCR-grade water. Thermal cycling conditions consisted of an initial denaturation at 95°C for 2min, followed by 30 cycles of denaturation at 95°C for 15 sec, annealing at 56°C for 20 sec, and extension at 72°C for 45sec. A final extension step of 5min at 72°C was included to complete amplification. To analyze PCR products, 6 µl of each amplified sample was resolved by electrophoresis on 1.5% agarose gels supplemented with RedSafe dye under a constant 100V for 30 min. Gels were visualized using a Bio-Rad Gel Doc XR system and product sizes estimated by comparison to a 100 bp DNA ladder.

2.8. Optimization of GNPs-assisted PCR

2.8.1. GNPs concentration optimization

To explore the impact of GNPs on PCR yield and sensitivity, a series of experiments were conducted. Various concentrations of GNPs, ranging from 0.4 nM to 3 nM, were introduced into the standard PCR reaction. The PCR reaction conditions, and program remained consistent across all experiments to ensure accurate comparisons. The resulting PCR products were meticulously analyzed and photographed, following the previously established methodology. This analysis enabled the assessment of how the presence of different GNPs concentrations influenced the amplification process. In addition to studying the effect of GNPs concentrations, the annealing temperature during PCR was also optimized. A range of annealing temperatures from 55℃ to 60℃ was tested to determine the most suitable temperature for efficient and specific PCR amplification.

2.8.2. Sensitivity

To compare the sensitivity of GNPs-assisted PCR and conventional PCR, 10-fold serial dilutions of the template enterotoxigenic *Bacillus cereus* DNA were used.

2.8.3. Specificity

To determine the specificity of the GNPs-assisted PCR assay, *Bacillus cereus* (ATCC11778) and 5 strai ns of nontarget bacteria (approximately 10^7cfu/ml): *B acillus subtilis* (ATCC 6633), *E. coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 6538), *Listeria monoc ytogenes* (ATCC 35125), and *Salmonella enteritidis* (MW 362308) were used.

2.9. Quantification of PCR product

. In this study, we employed the Bio-Rad Gel Doc XR documentation system software to quantitatively analyze the band intensities produced during the migration of the PCR amplified products on a 1.5% agarose gel. To achieve this, the ImageJ software was utilized to measure the intensity of each band accurately.

2.10. Real-time SYBRGreen(qPCR)

In this research, we conducted a SYBRGreen (qPCR) according to Wehrle et al. **[25]** using a 10 µl reaction mixture. The components of the reaction mixture included 5µl of the HERA PLUS SYBRGreen qPCR master mix, 0.4µl of a primer mix with a concentration of 10pmol, and 1µl of the DNA template. The reaction volume was brought up to 10µl using PCR-grade water to ensure accurate and consistent results. The qPCR program consisted of the following steps: initial denaturation at 95℃ for 2 min, followed by 40 cycles of denaturation at 95℃ for 15 sec, and annealing; extension at 60℃ for 20 sec. Additionally, a melting curve analysis was performed to further assess the specificity of the PCR products. Fluorescence parameters were collected after each annealing and extension step to monitor the amplification progress in real-time. The qPCR assay was carried out using the Step-One Applied Biosystems Real-time PCR System. This Real-time PCR platform offers high precision and sensitivity, enabling accurate quantification and monitoring of DNA amplification during the qPCR process.

Table 1: -Oligonucleotide primers specific for *nhe*A gene

gene	Primer sequence (5`→3`)	Product size	Reference
***nhe*A**	F-GTTAGGATCACAATCACCGC R-ACGAATGTAATTTGAGTCGC	755 bp	Guinebretière et al. 2002 **[32]**
	F-CTAAGGAGGGGCAAACAGAAG R- CGTATGCTTGAATAAGCGGTG	117 bp	Li et al.2016 **[33]**

3. Results

3.1. Characterization of the synthesized GNPs

The appearance of a ruby red color indicated the synthesis of GNPs. The average particle size was measured to be 12 nm with a PDI factor of 0.0843 **(Fig. 1A)**, and the surface charge was found to be negative polarity using DLS. The TEM image revealed the synthesized GNPs have a rather uniform size and spherical shape **(Fig.1B)**. Additionally, a λ_{max} specific peak at a wavelength of 520 nm was observed in the UV-visible spectroscopy **(Fig. 1C)**.

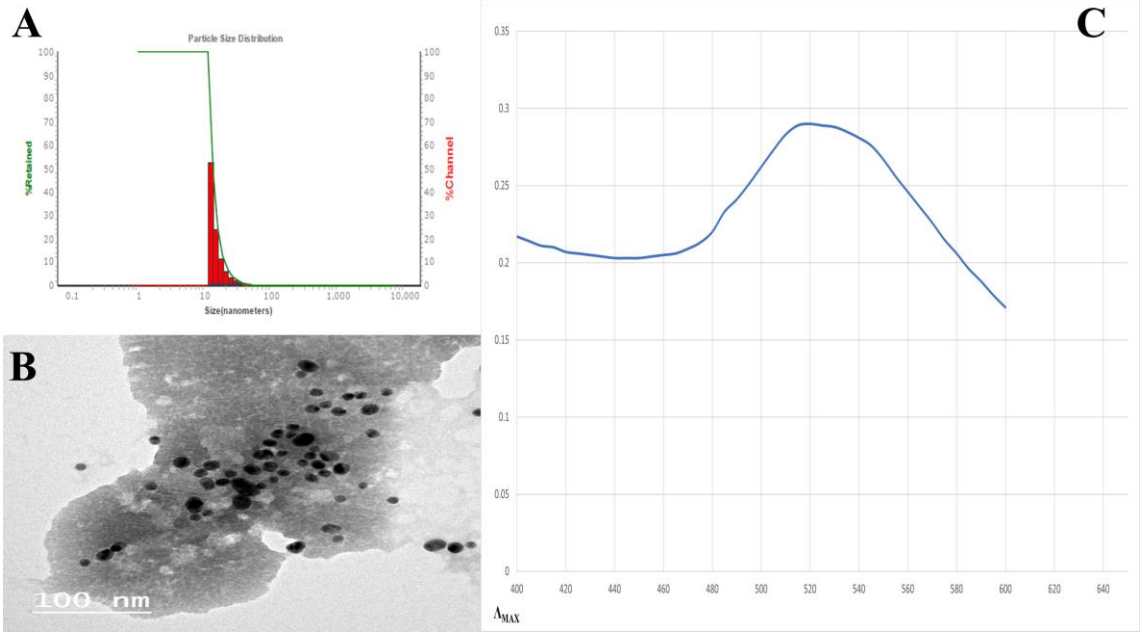

Fig.1. (A) The average particle size was 12nm. **(B)** TEM image of synthesized GNPs with spherical shape and reasonably uniform size. **(C)** A unique peak in UV-visible spectroscopy at 520 nm.

3.2. Sensitivity of conventional PCR

PCR demonstrated a detection limit of 10^3 copies of the *Bacillus cereus* DNA template, as determined through the amplification of 10-fold serial dilutions ranging from 10^7 to 10^1 copies with an optimized annealing temperature of 56°C **(Fig. 2)**.

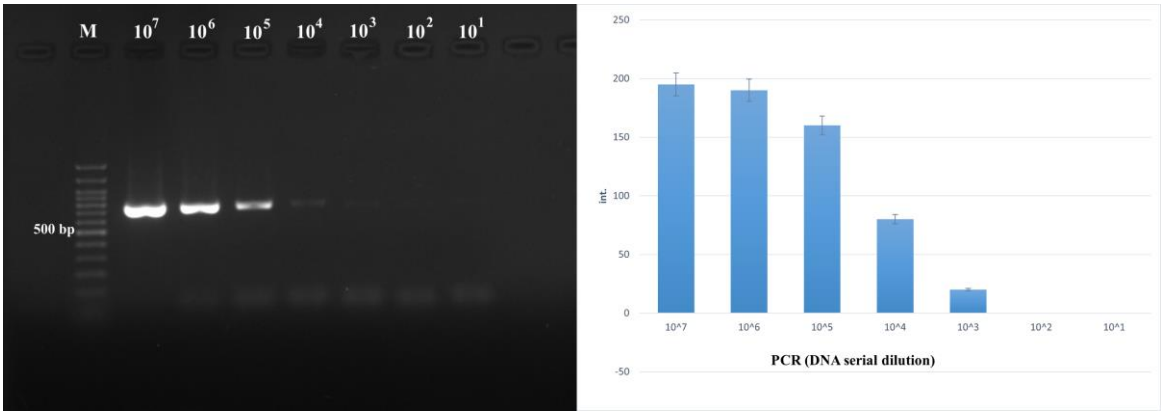

Fig.2. Conventional PCR sensitivity with 10-fold of DNA Serial dilution.

3.3. The effect of various GNPs concentrations on conventional PCR

PCR reactions carried out containing 0.4nM to 3nM GNPs using 10^5 copies of DNA template demonstrated that the yield of PCR is directly related to band intensity, establishing 1 nM as the optimal GNPs concentration for improved PCR sensitivity, as evidenced a rise in the PCR product band intensity on agarose gel **(Fig. 3)**.

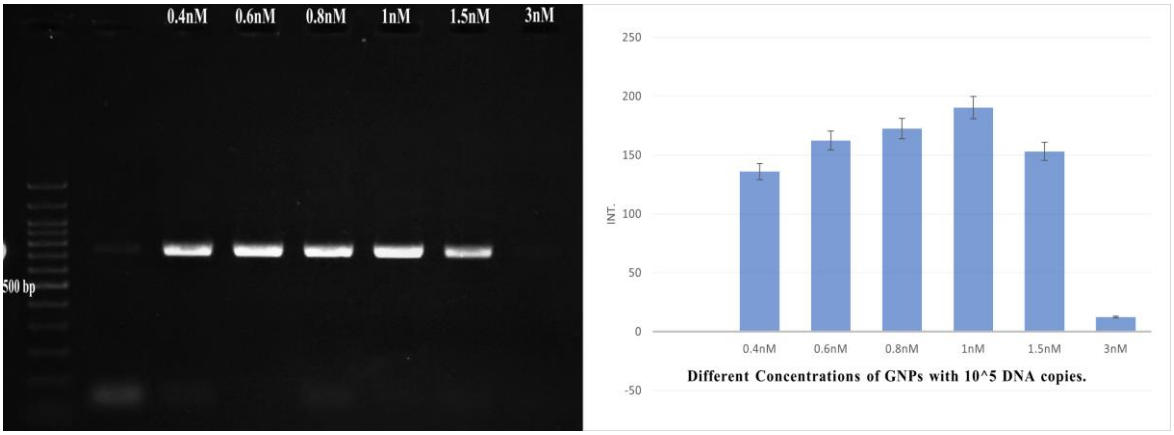

Fig.3. Different GNPs concentrations ranging between 0.4nM and 3nM with 10^5 copies of DNA template.

3.4. Sensitivity of GNPs-assisted PCR

To determine the GNPs-assisted PCR sensitivity, a 10-fold serial dilution of DNA template ranging from 10^7 to 10^1 copies was added to the PCR reaction with 1nM GNPs. The lowest number of target DNA copies that could be reliably detected using GNPs-assisted PCR was 100 copies of DNA **(Fig. 4)**. In comparison, the detection limit of conventional PCR without GNPs was 10^3 copies, as determined through experimental repetition.

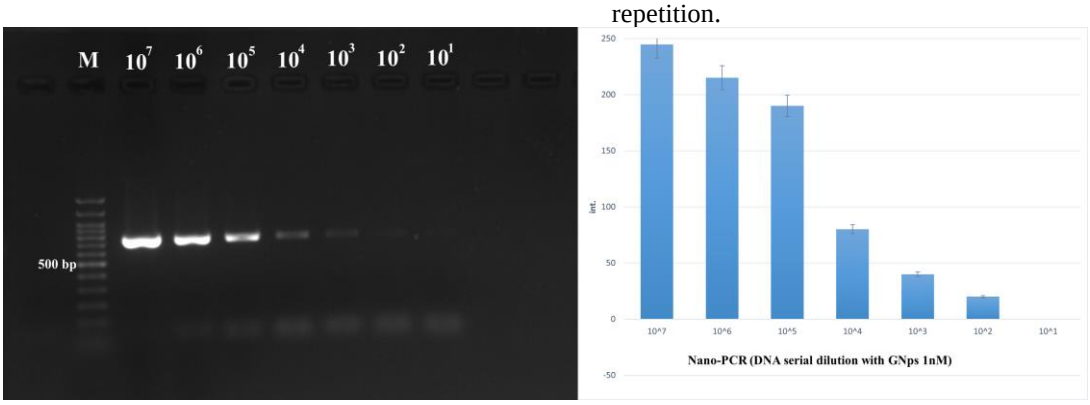

Fig. 4. GNPs-assisted PCR sensitivity with 10-fold of DNA Serial dilution and 1nM GNPs.

3.5. GNPs-assisted PCR vs. conventional PCR sensitivity for spiked milk samples

The influence of GNPs on PCR sensitivity was investigated through a series of experiments utilizing 10-fold serial dilutions of *Bacillus cereus* (ATCC 11778). Bacterial samples were subjected to DNA extraction followed by addition into the PCR mixture containing 1nM GNPs. For the study, a dilution series of *Bacillus cereus* ranging from 10^7cfu/ml to 10^1cfu/ml was prepared, alongside a negative control milk sample without spiking. Conventional PCR exhibited a detection limit of 10^3cfu/ml **(Fig.5A)**, while GNPs-assisted PCR demonstrated enhanced sensitivity with a lower detection limit of 10^2cfu/ml **(Fig.5B)**. These findings were replicated experimentally, further validating the improved sensitivity conferred by the GNPs-assisted PCR methodology. The serial dilution preparation, DNA extraction, PCR amplification, and replicate testing were systematically conducted to evaluate the effect of GNPs on PCR sensitivity in detecting *Bacillus cereus*. The results provide evidence that GNPs can lower the PCR detection limit 10-fold compared to conventional PCR, highlighting the sensitivity enhancing potential of GNPs-assisted PCR techniques.

The increase in sensitivity with GNPs-assisted PCR was also observed in spiked milk samples, with improved detection of 100 cfu/ml *Bacillus cereus* compared to 10,00 cfu/ml by conventional PCR **(Fig.5A, Fig.5B)**.

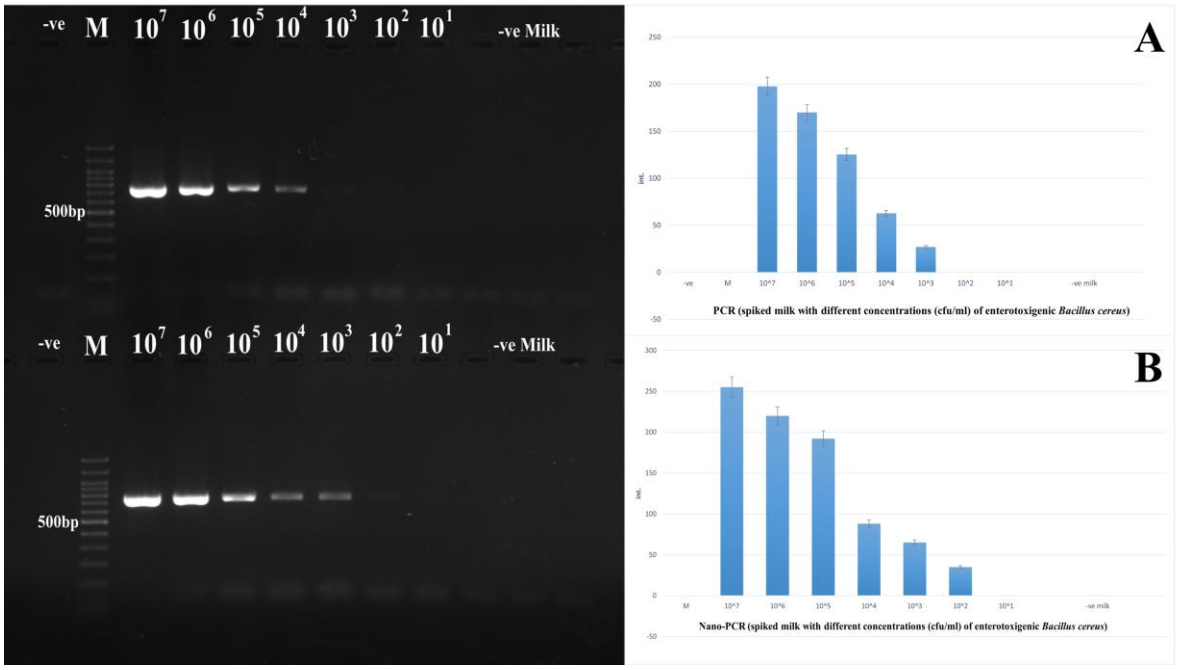

Fig.5. (A) PCR sensitivity on a 10-fold serial dilution of *bacillus cereus* from10^7cfu/ml to 10^1 cfu/ml spiked in milk samples. **(B)** GNPs sensitivity on the same dilution.

3.6. Sensitivity of SYBRGreen qPCR

In this study, we sought to evaluate the sensitivity of SYBRGreen qPCR through a series of experiments involving 10-fold dilutions of both the template DNA and *Bacillus cereus* (ATCC 11778). To assess the sensitivity of the qPCR method, we prepared a range of 10-fold dilutions of the template DNA, spanning from 10^7 to 10^1 copies. Subsequently, these diluted DNA samples were introduced into the PCR mixture to evaluate the qPCR's ability to detect varying DNA concentrations accurately. In addition to testing the DNA template, we also conducted 10-fold serial dilutions of *Bacillus cereus* (ATCC 11778). These dilutions covered a range from 10^7cfu/ml to 10^1cfu/ml, simulating different bacterial concentrations. DNA extraction on these diluted bacterial samples had been performed then introduced them into the PCR mixture to assess the qPCR's capability to detect *Bacillus cereus* at different concentrations. As a negative control, an unaltered milk sample was included in the analysis. Through repeated experimental trials, we obtained insightful results from the SYBRGreen qPCR. The qPCR method demonstrated a detection limit of 10^2 DNA copies as illustrated in (**Fig. 6A)** and a detection limit of 10^2cfu/ml for *Bacillus cereus* as shown in (**Fig.6B)**. The results demonstrated the remarkable sensitivity of SYBRGreen qPCR in detecting minute quantities of DNA and low levels of *Bacillus cereus*. The capacity to identify trace *Bacillus cereus* contamination highlights the utility of SYBRGreen qPCR for diverse applications including pathogen detection, environmental surveillance, and biomedical investigations. The robust performance for discerning low bacterial levels validates SYBRGreen qPCR as a valuable molecular tool for diverse sectors spanning clinical microbiology, food safety, environmental analysis, and life science research.

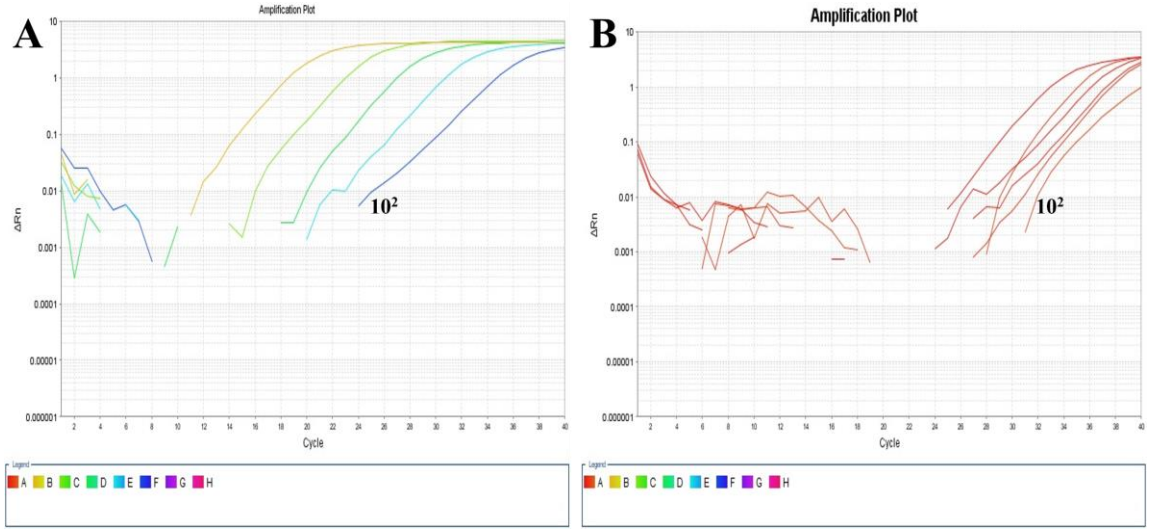

Fig.6. (A) Sensitivity of SYBRGreen qPCR on a 10-fold serial dilution of DNA, **(B)** 10-fold serial dilution of *bacillus cereus*.

3.7. Specificity

The specificity of GNPs-assisted PCR assay was evaluated by challenging it against *Bacillus cereus* (ATCC 11778) and 5 strains of non-target bacteria (approximately 10^7cfu/ml): *Bacillus subtilis* (ATCC 6633), *E. coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 6538), *Listeria monocytogenes* (ATCC 35125), and *Salmonella enteritidis* (MW 362308). The results demonstrated high specificity, with clear differentiation observed by the naked eye between enterotoxigenic *Bacillus cereus* and nontarget bacteria **(Fig. 7)**.

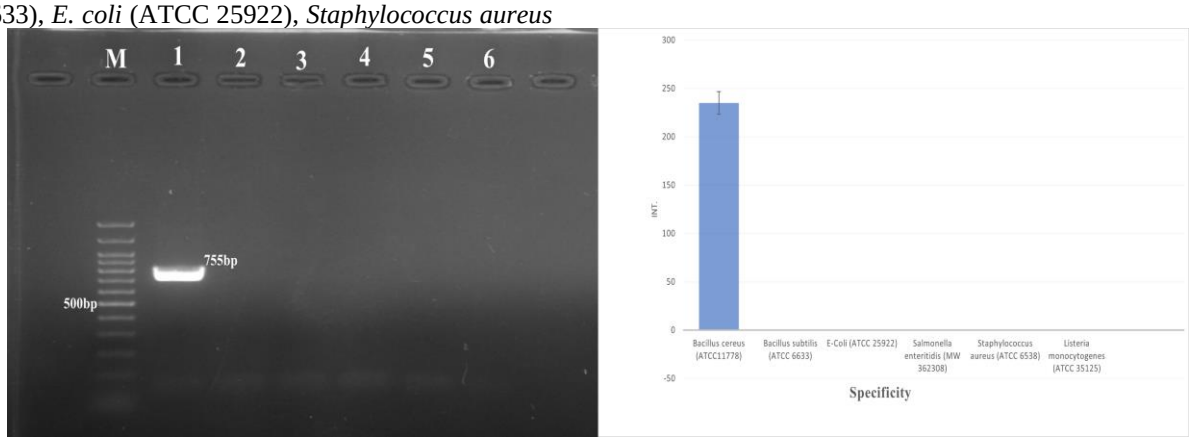

Fig.7. The specificity of GNPs-assisted PCR assay was evaluated by challenging against *Bacillus cereus* (ATCC 11778) and 5 strains of non-target bacteria: *Bacillus subtilis* (ATCC 6633), *E.coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 6538), *Listeria monocytogenes* (ATCC 35125) and *salmonella enteritidis* (MW 362308).

4. Discussion

Detection of enterotoxigenic *Bacillus cereus* in milk is imperative for food safety, as it can cause diarrheal food poisoning. *Bacillus cereus* generates several enterotoxins, with the nonhemolytic enterotoxin (*nhe*) being a major contributor to diarrhea **[35]**. The *nhe* genes are frequently detected in *Bacillus cereus* secluded from dairy products **[36,37]**. The present study demonstrated a 10^3copies DNA detection limit (Fig. 2) and 10^3cfu/ml limit (Fig. 5A) utilizing conventional PCR, aligning with other reported detection limits for *Bacillus cereus* **[15]**. However, some studies have reported superior detection limits. Hansen et al. **[38]** achieved 150 cfu/gm in spiked rice samples after overnight enrichment targeting the 16SrRNA gene. Kumar et al. **[39]** detected 10-100 cfu/ml in spiked milk and rice after overnight enrichment via multiplex PCR for enterotoxin genes. Propidium monoazide (PMA-multiplex) PCR targeting enterotoxin genes enabled Zhang et al. **[20]** to detect 22 cfu/gm in spiked milk powder after 3-4hrs enrichment. Yu et al. **[40]** attained

a 7.5x10^{2}cfu/ml limit in pure culture and 8 cfu/ml in spiked milk using multiplex PCR with enrichment.

Incorporating GNPs into PCR reactions can increase sensitivity compared to conventional PCR, likely due to the high surface area-to-volume ratio of GNPs concentrating PCR reagents **[41-43]**. Here, 12 nm GNPs were synthesized and characterized, displaying expected peaks by UV-vis spectroscopy and TEM (Fig. 1). Adding 1 nM GNPs boosted PCR yield (Fig. 3), permitting detection of 100 target gene copies versus 1000 copies with conventional PCR (Fig. 2,4).

The current study achieved a 100 cfu/ml detection limit for *Bacillus cereus* using GNPs-assisted PCR in spiked milk (Fig. 5B), 10-fold lower than the 1000 cfu/ml limit for standard PCR (Fig. 5A). These findings align with other reports on GNPs improving sensitivity, including 10^2 viral particle detection by GNPs-assisted PCR versus 10^4 particles without GNPs **[41]**, and 10-100 cfu/ml *Salmonella* detection by GNPs-multiplex PCR compared to 10^3cfu/ml without nanoparticles **[43]**. This likely involves concentrating PCR reagents on the high GNPs surface area.

Furthermore, SYBRGreen qPCR enabled a 100 copies DNA detection limit (Fig. 6A) and 100 cfu/ml *Bacillus cereus* detection limit (Fig. 6B), surpassing conventional PCR by 10-fold. Previous studies have applied SYBRGreen qPCR for sensitive *Bacillus cereus* and enterotoxin detection in foods **[25]**. Wehrle et al. **[25]** achieved 10 cfu/gm detection through a multiplex SYBRGreen qPCR assay for diarrheal enterotoxins. SYBRGreen facilitates target amplification monitoring and enhances sensitivity over conventional PCR. Fricker et al. **[36]** detected 10-100 cfu/gm by qPCR for the emetic toxin cereulide synthetase gene. However, SYBRGreen can bind nonspecifically, requiring melting curve confirmation **[44]**.

The GNPs-assisted PCR assay exhibited high specificity for *Bacillus cereus*, without non-specific amplification of non-target bacterial species, as evidenced by the results depicted in Figure 7. The assay targeting *Bacillus cereus* generated amplicons only for the *Bacillus cereus* samples, with no PCR products observed in reactions containing non-target bacterial DNA. This indicates the precision of the GNPs-assisted PCR method for selective identification of *Bacillus cereus* without cross-reactivity.

5. Conclusion

In conclusion, GNPs-assisted PCR targeting the *nhe*A gene significantly improves the detection sensitivity of enterotoxigenic *Bacillus cereus* in spiked milk samples. SYBRGreen qPCR provides a rapid and sensitive method for the detection of *Bacillus cereus* and its toxins in food samples. However, the increased sensitivity comes with the tradeoff of decreased specificity compared to probe-based methods. Additionally, qPCR requires more expensive instrumentation than standard PCR. The optimized GNPs-assisted PCR assay offers a good balance of sensitivity, specificity, cost, and ease of use for routine screening of *Bacillus cereus* in dairy products, enhancing food safety.

6. Conflict of interest

The authors declare no conflict of interest.

7. References

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