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Antimicrobial Peptide Magnetic Nanoparticles based Molecular Detection of *Bacillus cereus* in Milk and Milk Products

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ABSTRACT

Bacillus cereus is major spoilage microorganism in dairy products causing foodborne illness. In recent days, food safety is widely becoming an important public health issue, as foodborne diseases present a global public health problem. The rapid and precise monitoring and detection of foodborne pathogens are some of the most effective ways to control and prevent foodborne illness. In the present study, magnetic nanoparticles (MNPs) were prepared and characterized by particle size analyser, Zeta potential, X-ray diffraction (XRD) spectrophotometer analysis, Fourier Transform Infrared Spectroscopy (FTIR) and UV-visible spectrophotometer analysis. The results of the synthesized nanoparticles had particle size of 128 nm and zeta potential value of -50.8 ±2.2 mV. Crystal size of nanoparticle analysed by XRD was 51.24 nm. The magnetic nanoparticle were coupled with antimicrobial peptide cecropin and was utilized for entrapment of foodborne pathogens from milk and milk products followed by magnetic separation and PCR detection targeting HL gene of *B. cereus.* Further evaluation of nanoarticle entrapment and PCR, 150 food samples were screened for the presence of *B.cereus* in milk and milk products, which had improved detection sensitivity with 32% positivity in magnetic nanoparticle mediated entrapment followed by PCR when compared to 19% positivity in direct pelleting and PCR method.

Introduction

Food-borne diseases are mainly caused by pathogenic bacteria, which are transmitted to humans from the animal reservoir via food or which contaminates the food during processing (Olsen, 2000). *B. cereus* is a Gram-positive, aerobic or facultative anaerobic, rod-shaped, one of the most important endospore-forming spoilage microorganisms in dairy environment and also responsible for foodborne outbreaks around the world (Kumari and Sarkar, 2016). Species of *Bacillus* and related genera have long been troublesome to food producers on account of their resistant endospores. Their spores may be present on various types of raw and cooked foods and their ability to survive high cooking temperatures requires that cooked foods be served hot or cooled rapidly to prevent the growth of these bacteria (Anita and Swaid, 2015). It is essential to analyze the food for the presence of foodborne pathogens in order to ensure a safe food supply and to

minimize the occurrence of foodborne intoxications.

Traditional microbiological methods for identification of foodborne pathogens are time consuming and labor intensive and not suitable for rapid testing (Zhao *et al.,* 2013). Usually conventional methods require 2 to 3 days for preliminary identification and more than a week for confirmation of the species of the pathogens (Zhao *et al.,* 2014).

In this context, PCR is a rapid and sensitive technique that established as an alternative method for the conventional culture method for detection of bacteria in food (Wang *et al.,* 2009).

Molecular approaches targeting specific genes of pathogens utilizing DNA extracted from milk products have gained importance as they enable accurate detection but however with lesser sensitivity (Makino *et al.,* 1995). Further, researchers are still developing novel methods with improvements in terms of rapidity, sensitivity, specificity and suitability for *in situ* analysis and distinction of the viable cell (Zhao *et al.,* 2014).

Rapid detection methods are important, particularly in food industry, as they are able to detect the presence of pathogens in raw and processed foods immediately. Rapid methods are also sensitive enough to detect pathogens that present in low numbers in the food. Sensitivity is important because a single pathogen present in food has the risk to cause infection. However, application of molecular biological techniques like Polymerase Chain Reaction (PCR) on complex sample matrices such as milk is always accompanied several inhibitors and had lower sensitivity and exhibit negativity when pathogens are in low numbers. Recently, magnetic nanoparticles are widely being applied for the specific enrichment of target cells from the complex matrices, due to their strong external magnetic field response and thereby improve the efficiency of detection techniques, (de Dios and Diaz Garcia, 2010). Nanoparticles enable easy and reliable application that does not require any expensive equipment's, reduce the test detection time, and also use simple methodologies making them suitable for rapid detection assays.

The combination of nanotechnology with the analytical method helps to improve the sensitivity of the assay, reduce the running cost and time (Alharbi and Al-Sheikh, 2014). In this study, magnetic nanoparticles were synthesized, characterized coupled with antimicrobial peptide cecropin and utilized for bacterial entrapment from food matrices followed by their detection by PCR. The efficacy of the particle entrapment and detection sensitivity of was determined and the nanoparticle enabled rapid and sensitive detection of bacterial pathogens from various food matrices.

Materials and Methods

Preparation of iron oxide nanoparticles and characterization

Magnetic iron oxide nanoparticles were synthesized by chemical co-precipitation method using 0.01 M iron (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) and 0.02 M iron (II) chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$) in triple distilled water. The mixture was kept at 60-80°C for 15 mins with constant stirring. 0.8 N NaOH was added to the mixture at constant flow rate and the mixture was stirred continuously for 3 hours at 60-80°C and the magnetic nanoparticles were separated with a magnet.

Further, magnetic nanoparticles were washed twice with triple distilled water and once with ethanol. Finally, the nanoparticles were dried

in hot air oven and fine powdered using mortor and pestle. The synthesized magnetic iron oxide nanoparticles were analyzed for their particle size using SZ100 particle size and zeta potential analyzer (Horiba, Japan). The crystalline or amorphous nature of magnetic iron oxide nanoparticles were characterized by XRD analysis. The X-ray diffraction (XRD) patterns were recorded using a Scintag 2000 PDS diffractometer with Cu K∞ radiation with the 2θ range of 0-70º. XRD patterns were calculated using X'per Rota flex diffraction meter using Cu K radiation and λ =1.5406 A°. Crystallite size is calculated using Scherrer equation. The surface modification and Surface Plasmon Resonance property was characterized by FTIR and UV-visible spectrophotometer analysis.

Coupling of antimicrobial peptide to magnetic nanoparticles

Coupling of magnetic nanoparticles with antimicrobial peptides were carried out by addition of 50 µl (25mg/ml) of each nanoparticles solution with 1.0 µl of cecropin antimicrobial peptide, 2.5 µl of 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (0.4 M) and 50 µl of N-Hydorxysuccinamide (NHS)(0.1 M) in 450 µl phosphate buffer (10 mM, pH-7.4) and incubated at 30 rpm for 1 hour and 30 minutes (Vashist, 2012). Then 50 µl of 10 mM sodium bicarbonate buffer (pH 8.8) containing 10% bovine serum albumin (BSA) was added and incubated at 30 rpm for 30 minutes.

Finally, the coupled magnetic nanoparticles were separated using permanent magnet and washed twice with 10 mM sodium bicarbonate buffer (pH 8.8) containing 0.1% BSA. The pellet was finally resuspended in 200 µl of sodium bicarbonate buffer (10 mM, pH 8.8 with 0.1% BSA) and utilized for further studies for bacterial entrapment.

Entrapment of bacteria using antimicrobial peptide coupled magnetic nanoparticles and PCR detection

B.cereus isolate (ATCC 117783) was plated in selective agar and confirmed by cultural characterization and biochemical techniques. A single colony is inoculated in 2ml of broth and pasteurized milk samples, incubated for 6 hrs. From this tenfold serial dilution is performed and colony forming units per millilitre (CFU/ml) was determined. To all the serial dilutions 3% w/v of magnetic particle is added and the capture efficiency of magnetic nanoparticle is determined by plating in selective agar and CFU/ml was calculated.

The difference in CFU/ml of the original spiked known count of bacteria and the captured supernatant provided the binding efficiency of the magnetic particle in broth as well as in pasteurized milk samples. The antimicrobial peptide coupled magnetic nanoparticles were used for bacterial entrapment in each of the serial dilutions for detection of foodborne pathogen *B.cereus* in milk and milk products. For capturing of foodborne pathogens, 100 µL of overnight culture was mixed with of antimicrobial peptide coupled magnetic nanoparticles at a level of 3% w/v.

The mixture was incubated in a shaking incubator with 130 rpm at 37°C for 30 mins to allow the binding of antimicrobial peptide to the bacterial surface. After incubation, the tube was placed in a magnetic separator. Finally, the supernatant was discarded and the DNA was extracted from the pellet by boiling method.

The final pellet was suspended in 20µl of Nuclease free water (NFW) and boiled for 10 minutes, centrifuged at 10000 rpm for 3 minutes and the lysate was used as DNA template for PCR.

PCR and its detection sensitivity in spiked milk samples

The DNA lysate from the samples was used as a template for further confirmation of the presence of foodborne pathogen *B.cereus* by PCR using gene specific primers set as forward primer, FP:TTCGGCTCCACCT GTTATG and reverse primer, RP: TCGCAT CTCCACCTAATACG designed in the study, with optimized primer annealing temperature as 55°C targeting 618 bp haemolysin gene of *B. cereus.* PCR amplification was performed in a total reaction volume of 25µl with 12.5 µl of master mix, 20 pmol of the forward and reverse primer and the template DNA.

The PCR cycle condition for detection of *B.cereus* is 94°C for 10 min, 35 cycles of 95°C for 2 min, 58°C for 45 sec, 72 °C for 4 min and final extension of 72 for 10 min. The PCR products were separated by 1.5% gel electrophoresis, ethidium bromide stained and visualized by UV illumination.

To determine the detection sensitivity of PCR after bacterial entrapment, here we spiked known count of *B.cereus* in tenfold serial diluted pasteurized milk samples and entrapped by 3%w/v of magnetic nanoparticles, incubated for 30 min.

The samples were processed by direct pelleting at 10000 rpm for 10 min in first method and secondly by magnetic separation mediated entrapment. The pellets were used for DNA extraction by boiling method. The total DNA lysate were utilized in PCR amplification and the results were analysed by agarose gel electrophoresis.

Detection sensitivity of PCR on magnetic separation on screening various dairy products

To determine the applicability of the magnetic nanoparticles in entrapping bacterial pathogen from food matrices, a total of 150 milk and milk products including raw milk, pasteurized milk, flavoured milk khoa, butter, cheese and paneer were utilized. These samples were processed for bacterial entrapment, magnetic separation and followed by PCR detection targeting 618 bp haemolysin gene of *Bacillus cereus.*

Results and Discussion

Rapid and sensitive detection of food borne pathogen is the need of the hour for effective control of food borne intoxication. Various methods including conventional cultural and molecular based assays have been used in detection methods from complex food matrices with varying sensitivity, accuracy, need for expensive equipment and time period. In recent past, combination of nanotechnology with different analytical assays are widely developed and utilized for rapid detection of food pathogen. It is known that, Gram positive bacterial membranes are made up of thick Peptidoglycan layer rich in Teichoic acids that have phosphate groups in their structure which contribute to the negative charge. The cationic property of the antimicrobial peptide due to the presence of amino groups on their surface, the cecropin coated magnetic nanoparticle imparts a positive charge to the particle which results in electrostatic interaction between the nanoparticle and the bacterial pathogen. Based upon the fact, here we report a rapid and improved method in detecting food pathogen from various milk and milk products by PCR using antimicrobial peptide coupled magnetic nanoparticle bacterial entrapment. Here, magnetic nanoparticles were synthesized and analyzed for their particle size using SZ100 particle size and zeta potential analyzer (Horiba, Japan) which was 170.4 nm (Fig. 1). The magnetic iron oxide nanoparticles had good stability with the zeta potential value of -50.8 ±2.2 mV. The

XRD analysis indicated that the magnetic iron oxide nanoparticle was crystalline in nature with an average size of 51.24 nm, (Fig. 2).

FTIR measurements were carried out to identify the possible biomolecules responsible for reduction and capping of iron oxide nanoparticles. The absorbance maxima were scanned by FTIR at the wavelength of 400-4000 cm^{-1}. Based on the results the broad bands of 3335 and 3332 cm^{-1} of magnetic iron oxide nanoparticles were related with γ(OH) which is responsible for reduction of nanoparticles. The Fe-O peak was recorded at 586 cm^{-1} (Fig. 3). The Surface Plasmon Resonance property of synthesized nanoparticles was confirmed by UV-visible spectrophotometric analysis with the wavelength range of 200-800 nm. The maximum absorbance peak was observed at 250 nm for the magnetic nanoparticles (Fig. 4).

Table.1 Dairy products screened for *Bacillus cereus*

S. No.	Samples	No. of samples screened	No. of samples positive magnetic separation and PCR	No. of samples positive by direct pelleting and PCR
1.	Raw milk	30	17	11
2.	Pasteurized milk	50	12	9
3.	Khoa	25	6	2
4.	Butter	10	4	1
5.	Paneer	15	4	2
6.	Flavoured milk	10	2	2
7.	Skim milk	10	3	2
	Total	**150**	**48**	**29**

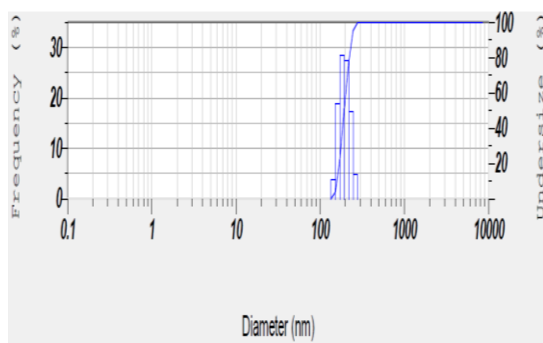


Fig 1: Particle size of magnetic iron oxide nanoparticles

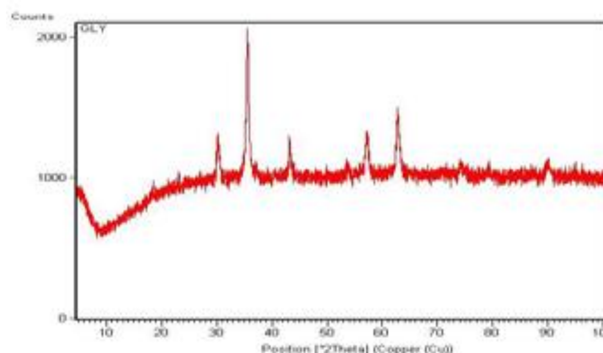


Fig.2: XRD pattern of magnetic iron oxide nanoparticles

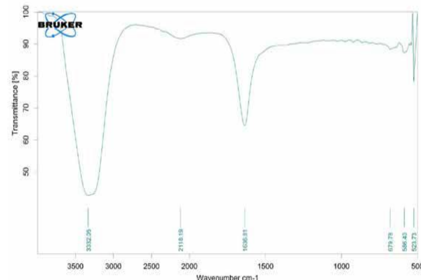


Fig.3: **FTIR spectra of magnetic nanoparticles**

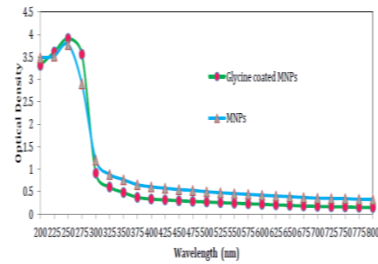
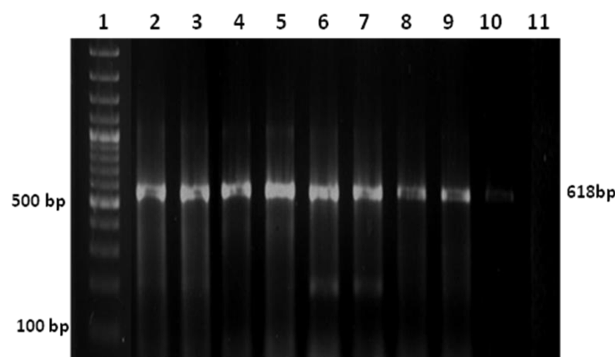


Fig.4: **UV-visible spectrophotometer analysis**

Plate.1 PCR amplicons of 618 bp haemolysin (HL) gene. Lane 1: 100 bp ladder, Lane 2-10: *Bacillus cereus* amplicons from dairy products Lane 11: Non template control (NTC)



Coupling of magnetic iron oxide nanoparticles with antimicrobial peptides were carried out and the coupling efficiency was found to be 92.32%.The AMP coupled magnetic nanoparticle was used for bacterial entrapment from food matrices and followed by analytical detection assays. Recently colloidal gold labelled AMP as probes in combination with specific antibodies was developed to detect shiga toxin producing *E.coli* in a lateral flow assay (Yonekita *et al.,* 2013). Many researchers use other biomolecules to replace antibodies to prepare magnetic separation probes, such as vancomycin, D - mannose (El-Boubbou *et al.,* 2007) and aptamer (Bruno *et al.,* 2014). Bai *et al.,* 2013 used amino modified silica coated magnetic nanoparticles (ASMNPs) that could adsorb one or more types of pathogens from milk samples without use of biomolecules. Here, we attempted to use antimicrobial peptide (AMP) coupled magnetic nanoparticle for bacterial entrapment of pathogens from milk and milk products followed by PCR amplification in rapid and precise detection of *B.cereus.*

In the present study, the results revealed that the binding efficiency of magnetic nanoparticle for bacterial entrapment in broth and milk sample at the highest dilution was 78.4% and 71.7% respectively. Also the binding efficiency at the lowest dilution was 59.4% and 51.6% in broth and milk samples respectively, indicating that AMP coupled magnetic nanoparticle could entrap around 52% pathogen in complex milk samples. Venkatesan *et al.,* 2014 observed a binding efficiency of 74.2% and 65.9% for high biofilm, 61.5% and 44.2% for low biofilm of

Staphylococcus aureus and 48.5% and 69.4% for *E. coli* in broth and milk samples respectively. Also at higher dilutions, the binding efficiency may be decreased due to the lower levels of bacteria in milk and milk products and also their lower growth rate. The binding efficiency may vary for different bacteria due to various factors including their growth rate, sample matrices, adherence and biofilm formation capacity of the bacteria.

Detection sensitivity of PCR on magnetic separation in spiked samples

PCR efficiency following bacterial entrapment and magnetic separation and its utility in direct detection of food pathogens was evaluated in milk samples spiked with *B.cereus* in ten fold serial dilutions in milk. The detection limit of *B.cereus* was 2.88 x 10^6 CFU /ml in direct pelleting of milk samples followed by PCR. However, in magnetic nanoparticle mediated followed by PCR by magnetic particles from milk samples the detection limit was 1.2 x 10^2 CFU /ml, indicating that the presence of as low as 100 bacteria could be detected precisely. Lee *et al.,* 2014 used a differently modified magnetic mesoporous silica particle for capture of *E. coli* 0157:H7 strain from milk and *Staphylococcus aureus* in broth samples and reported a sensitivity of 1.1 x 10^6 to 1 log10 CFU/ ml when detected by realtime PCR assay.

Detection sensitivity of PCR on magnetic separation on screening various dairy products

Magnetic nanoparticles entrapment and PCR enabled rapid detection of *B.cereus* form various dairy products. Out of 150 samples screened, the direct pelleting followed by PCR could detect only 29 samples, whereas the AMP coupled magnetic nanoparticle mediated entrapment followed by PCR detected 48 samples for the presence of *B.cereus.* The prevalence of *B.cereus* in ready to eat dairy products was 19% by direct pelleting followed by PCR and 32% by using magnetic entrapment of bacterial pathogen and polymerase chain reaction detection. Here, 48 dairy samples were confirmed by PCR targeting haemolysin toxin gene that produced 618 bp amplified products (Plate 1) indicating that the ready to eat milk products as a significant source of *B.cereus* contamination with toxin production (Table 1). These results indicate that antimicrobial peptide (AMP) coupled magnetic nanoparticle mediated bacterial entrapment had improved sensitivity in PCR detection and hence could detect *B.cereus* in complex food matrices in shorter duration. The overall detection assay time involved was 12 hours, including 6 hrs incubation, 1 hr magnetic separation, followed by 5 hrs of PCR and post PCR assay.

In conclusion the antimicrobial peptide coated magnetic nanoparticles were successfully synthesized and utilized for detection of *B.cereus* from milk and milk products. The developed method is rapid, sensitive, avoids overnight bacterial enrichment and incubation procedure and resulted in rapid detection of *B.cereus.* Further, the nanoparticle based direct PCR assay need to be assessed for its detection sensitivity and specificity in separating *B.cereus* pathogen and other food pathogens from various food matrices in larger numbers to determine its wider applicability in rapid diagnostics of food pathogens in ensuring food safety.

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Conflict of interest statement

The author expresses no conflict of interest with any other individual or organisation regarding the information discussed in the manuscript.

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