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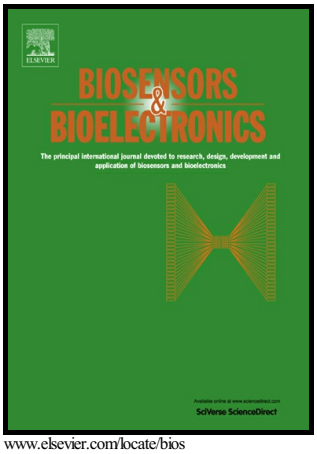

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Rapid detection of *Salmonella enterica* in food samples by a novel approach with combination of sample concentration and direct PCR

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Abstract

Foodborne salmonellosis remains a major economic burden worldwide and particularly for food industries. The diverse and complexity of food matrices pose great challenges for rapid and ultra-sensitive detection of *Salmonella* in food samples. In this study, combination of pathogen pre-concentration with rapid molecular identification is presented to overcome these challenges. This combination enabled effective real-time PCR detection of low levels of *Salmonella enterica* serovar Typhimurium without culture enrichment. Anti-salmonella antibody, immobilized on protein AG-magnetic beads, could efficiently concentrate *Salmonella* Typhimurium with a capturing efficiency of 95%. In the direct PCR, a strong linear relationship between bacteria concentration and the number of cycles was observed with a relative PCR efficiency of ~92% resulting in a limit of detection (LoD) of ~2 CFU/mL. Analysis of spiked food samples that include vegetable salad, egg yolk, egg white, whole egg and minced pork meat has validated the precision of the method. A relative accuracy of 98.3% with a sensitivity of 91.6% and specificity of 100% was achieved in the *Salmonella* spiked food samples. The use of a Phusion hot start DNA polymerase with a high tolerance to possible PCR inhibitors allowed the integration of direct PCR, and thereby reducing the duration of analysis to less than 3 hours. The Cohen's kappa index showed excellent agreement (0.88) signifying the capability of this method to overcome the food matrix effects in rapid and ultra-sensitive detection of *Salmonella* in food. This approach may lay a future platform for the integration into a Lab-on-a-chip system for online monitoring of foodborne pathogens.

Keywords: Sample concentration, *Salmonella* detection, Direct PCR, Immuno-magnetic beads, Food safety

1. Introduction

Non-typhoidal *Salmonella enterica* is one of the leading causes of foodborne illness worldwide and represents a considerable burden in both developing and developed countries (EFSA and ECDC, 2018; WHO, 2018). Gastroenteritis and diarrheal diseases due to *Salmonella* species accounts for 94 million cases and 155,000 deaths globally every year (Majowicz et al., 2010). Salmonellosis is the second most commonly reported foodborne infection in the European Union (EU) and in the United States (US). European Food Safety Authority (EFSA) has estimated that over 90,000 salmonellosis cases are being reported every year in the EU, resulting in an overall economic burden as high as EUR 3 billion a year (ECDC, 2018; EFSA and ECDC, 2017). In the USA, *Salmonella* species accounts for approximately 34% of the outbreaks imposing an estimated economic burden of $3.7 billion in a typical year (CDC, 2017; Hoffmann et al., 2015). More than 75% of the *Salmonella* outbreaks were attributed to seeded vegetables, egg, chicken, pork, beef, or vegetable row crops (IFSAC, 2017). Conventional bacterial culture methods of *Salmonella* detection in food samples require time-consuming enrichment step followed by selective plating, biochemical and/or serological identification. These techniques are not ideal for products with an inherently short shelf life e.g. salad, egg or ready to eat foods that are minimally processed (López-Campos et al., 2012). In recent years, several real time quantitative PCR (qPCR)-based techniques have been developed to detect *salmonella* in various food samples as an alternative (Almeida et al., 2013; Schuurman et al., 2007). DNA purification being the prerequisite to the qPCR is a time-consuming step and may compromise with sensitivity due to recovery loss. Inhibition of qPCR originating from the food sample presents additional concerns that can drastically reduce the sensitivity and the amplification efficiency and thereby affecting the accuracy and quantification (Rådström et al., 2008). In this direction, Phusion high-fidelity DNA polymerases (*Pfu*) has emerged as a potential alternative to overcome PCR inhibitors (Wang et al., 2004). *Pfu* DNA polymerase that has been fusion with a double-strand DNA-binding domain (Sso7d) show good processivity, high catalytic activity and enzyme stability. The Sso7d protein improves the performance of the *Pfu* DNA polymerase by

guiding negative supercoiling (López-García et al., 1998), increasing the tolerance to high salt concentration and other PCR inhibitors. The feasibility of this reengineered DNA polymerase has recently been demonstrated for detecting pathogens in food and animal samples at slaughter (Chin et al., 2017).

The diverse and complexity of food matrices may also pose great challenges for the development of a rapid and ultra-sensitive detection methods. Sample preparation is the most challenging problem in the direct food sample analysis. This may be due to the heterogeneity of the food matrices, the non-uniform distribution of pathogens and low abundance of target pathogens in the food. In addition, the presence of indigenous microflora can often interfere with selective identification and limited measurement volumes can limit the sensitivity (Kretzer J.W., Biebl M., 2008; Mandal et al., 2011). Therefore, sample preparation and concentration process is the most critical step concerning the applicability of novel methods. In this direction, immunomagnetic separation method have been applied for selective concentration of target bacterial pathogens in order to eliminate the effects of PCR inhibitors and to reduce the heterogeneity of food samples (Fedio et al., 2011; Hyeon and Deng, 2017; Zheng et al., 2016, 2014). However, in these studies, a minimum of 4 hrs of culture enrichment followed by DNA extraction or complex multi step approach were adopted.

In response to the demand for rapid analysis and simple strategy suitable for at-site detection of pathogens, in this study, a novel approach of combination of sample concentration and Phusion DNA polymerases-based direct PCR is described. Protein A/G was used as a bio-adapter to immobilize anti-salmonella antibodies on the magnetic beads to concentrate *Salmonella* directly from the food samples. The use of protein A/G for controlling the orientation of antibodies in order to maximize the capacity of pathogen recognition with high sensitivity is emphasized. The performance of the direct PCR on the pathogen captured magnetic beads without DNA extraction and pre-PCR purification is evaluated.

2. Methods

2.1. Optimization of immuno-magnetic bead concentration for direct PCR

Immuno-magnetic bead conjugate was prepared by immobilizing anti-salmonella antibodies on the protein A/G magnetic beads according to the manufacturer's instructions (See *supplementary materials* for details). The direct PCR was performed on 0.1 μg, 1 μg, 10 μg and 100 μg of anti-salmonella antibody-bead conjugates (Ab-bead) in separate experiments using Phusion Human Specimen Direct PCR Kit in 10 μL reaction volume. Initially, the Ab-bead conjugates were transferred to PCR tubes and mixed with 6 μL of a direct PCR mixture containing 200 nM of *hil*A primers (F: 5'-GCGACGCGGAAGTTAACGAAGA-3', R: 5'-GCAGACTCTCGGATTGAACCTGATC-3') targeting the transcriptional regulator *hil*A gene of *Salmonella* Typhimurium (CCUG – 31969), 1X Phusion® human specimen PCR buffer, 0.04 U/μL Phusion human specimen DNA polymerase and 2X SYBR® Green DNA intercalating dye (Chin et al., 2017). Prior to the PCR, an overnight culture of *Salmonella* Typhimurium (10^4 CFU in 4 μL) were added. The real-time PCR was conducted in Mx3005P qPCR Systems (Agilent Technologies, USA) under the PCR condition of 98 °C for 10 min following by 38 cycles of 98 °C for 10 s, 60 °C for 15 s and 72 °C for 30 s. Further, the PCR amplified products were confirmed by 2% agarose gels electrophoresis containing 1X SYBR® Safe DNA gel stain (Invitrogen, Life Technologies, USA) and the gel image captured (Gel Doc 2000, Bio-Rad) was assessed by ImageJ software (https://imagej.nih.gov/ij/).

2.2. Studies on the direct PCR assay efficiency and sensitivity

2.2.1. Salmonella concentration and the estimation of capturing efficiency

An immuno-magnetic bead-based *Salmonella* concentration method was developed in the present study (Scheme 1) and the capturing efficiency was estimated. 10 μg of the Ab-bead conjugate that corresponds to ~$5*10^6$ beads (See *supplementary materials* for details) were mixed with $2.4*10^4$ CFU of *Salmonella* Typhimurium spiked in 1 mL of 10 mM PBST. The mixture was incubated at 37 °C for 30 minutes and concentrated using the magnetic stand. The supernatant was preserved and 100 μL of 1:10 dilution was plated on a BA plates in duplicates and incubated overnight at 37 °C. The concentrated bead-*Salmonella* Typhimurium complex was washed 4 times with 1 mL of PBST. 100 μL of each

washing solution were plated directly on BA plates. The efficiency of Ab-bead to capture *Salmonella* was estimated by bacterial culture colony count (CFU) method.

2.2.2. Evaluation of assay sensitivity and PCR efficiency

In order to evaluate the direct PCR efficiency, initially, a standard graph of *Salmonella* Typhimurium was generated by qPCR method. A serial 10-fold dilution of *Salmonella* Typhimurium in 1 mL of PBS was prepared from a stock culture ($O.D_{600}$ = 0.8, corresponding to $5.64*10^8$ CFU/mL as calculated by colony counting method). One µL of each dilution of *Salmonella* Typhimurium was added to a PCR solution containing 10 µg of ab-bead conjugate (10 µL final volume) to give a final concentration ranging from 6 to $5.64*10^5$ CFU/10 µL PCR reaction in triplicates. The Direct PCR was conducted as mentioned earlier. Ct values (cycle threshold) obtained for each concentration was plotted against the $\log_{10}$ concentration (CFU) of *Salmonella* Typhimurium.

To test the sensitivity of the developed integrated immuno-magnetic direct PCR method, serial dilution of *Salmonella* Typhimurium cells were spiked in 1 mL of 10 mM PBST to give a final concentrations ranging from 6 to $6.6*10^4$ CFU/mL in triplicates. A non-spiked sample was included as a negative control. The spiked pathogen samples were concentrated using 10 µg of Ab-bead conjugate and the direct PCR was conducted on these beads as mentioned in previous section (Scheme 1). The Ct values were plotted against the $\log_{10}$ of concentration of *Salmonella* Typhimurium spiked in the PBS. Further, the amplified PCR products were confirmed by 2% agarose gels electrophoresis. The Direct PCR amplification efficiencies (*E%*) for both the controls and the magnetically concentrated samples were determined based on the slope of the standard graph according to the following standard equation (Fraga et al., 2012);

$$E(\%) = [10^{\left(\frac{-1}{slope}\right)} - 1] * 100 \qquad \textit{(Eq: 1)}$$

The average recovery (%) of *Salmonella* Typhimurium from the spiked PBS samples was determined by interpolating the Ct values on the *Salmonella* Typhimurium standard

graph. The capturing efficiency of ab-bead conjugate was estimated according to the equation S1 (*supplementary material*).

2.3. Analysis of Salmonella spiked food samples

2.3.1. Food sample preparation and optimization of sample dilution conditions

Fresh food samples that include mixed vegetable salad, chicken egg and minced pork meat were purchased from the local supermarket in Denmark. The samples were processed according to Nordic Committee on Food Analysis reference method (NMKL-71, http://www.nmkl.org/index.php/en/webshop/item/salmonella-pavisning-i-livsmedel-nmkl-71-5-utg-1999) with slight modification. Initially, 25 g of mixed vegetable salad, egg yolk and whole egg samples were homogenized in 25 mL of Buffered Peptone Water (BPW, 1:1 dilution) for 10 minutes. A minced pork meat sample was homogenized in 125 mL of BPW (1:5 dilutions). After thorough mixing, the mixed vegetable salad and the minced pork meat samples were filtered separately using filter stomacher bags (BagPage®R, Interscience, France). The respective debris free food matrix solutions was carefully collected. The mixed vegetable salad matrix was preserved till further use at 4 °C.

In order to observe the effect of sample dilution on the efficiency of *Salmonella* concentration and direct PCR, a *Salmonella* Typhimurium culture of 770 CFU in 100 μL (as calculated by colony counting) was spiked into 250 μL, 500 μL and 750 μL of the minced pork meat, the egg yolk, the whole egg and an undiluted egg white samples separately. The volume of the spiked samples was adjusted to 1 mL with 10 mM PBST. The spiked *Salmonella* Typhimurium cells were concentrated from each samples separately using 10 μg of Ab-bead conjugate and the direct PCR was conducted on these beads as mentioned above. Amplified PCR products were confirmed by electrophoresis using 2% agarose gel.

2.3.2. Studies on the limit of detection of immuno-magnetic bead combined direct PCR method in the Salmonella spiked food samples

To determine the limit of detection in the spiked food samples, a 10-fold serial dilution of *Salmonella* Typhimurium was prepared from a stock culture ($O.D_{600}$ = ~0.8). 100 μL of each dilution was spiked into 200 μL of the mixed vegetable salad (1:5 dilution) and 100 μL of the minced pork meat, the egg yolk, the whole egg and the undiluted egg white samples (1:10 dilution) separately in triplicates. The volume of the spiked samples was adjusted to 1 mL with 10 mM PBST to attain a final concentration gradient of *Salmonella* cells ranging from 10^0 to 10^4 CFU/mL. A non-spiked sample was included as a negative control for each sample. The spiked pathogens were concentrated and the direct PCR was conducted as mentioned in previous section. PCR amplified products were confirmed by 2% agarose gels electrophoresis and the efficiencies (*E%*) of the Direct PCR amplification were determined according to the *Eq. 1* for each samples.

2.4. Evaluation of assay precision and statistical analysis

Concentrations of *Salmonella* Typhimurium recovered from the spiked food samples were calculated by interpolating the mean Ct values of each trial on the standard curve. The efficiency of recovery (%) was estimated by comparing the calculated concentration with the spiked concentration over the concentration gradient for each sample. Inter assay precision and repeatability of the developed technique was determined by calculating coefficient of variance (CV%). Relative sensitivity, relative specificity and relative accuracy of the integrated immuno-magnetic direct PCR assay in the food samples were statistically evaluated based on the following relations (Chin et al., 2017)

$$Relative\ accuracy\ (AC\%) = \left[\frac{(\mathrm{PA}+\mathrm{NA})}{\mathrm{N}}\right] * 100 \qquad \textit{(Eq: 2)}$$

$$Relative\ specificity\ (SP\%) = \left[\frac{\mathrm{NA}}{\mathrm{N}-}\right] * 100 \qquad \textit{(Eq: 3)}$$

$$Relative\ sensitivity\ (SE\%) = \left[\frac{\mathrm{PA}}{\mathrm{N}+}\right] * 100 \qquad \textit{(Eq: 4)}$$

PA: Positive agreement (recovery) between the PBS and food samples
NA: Negative agreement (recovery) between the PBS and food samples
N: Total number of samples (NA+PA+PD+ND)

PD: False positives recovery from food samples

ND: False negatives recovery from food samples

N-: Total number of negative results (NA+PD)

N+: Total number of positive results (PA+ND).

Cohen's kappa inter assay agreement was assessed between the presence and absence of different food matrix as given below (Carpentier et al., 2017; Hyeon and Deng, 2017);

$$Cohen's\ Kappa\ index = \left[\frac{\mathrm{P}(o)-\mathrm{P}(e)}{1-\mathrm{P(e)}}\right] \qquad \textit{(Eq: 5)}$$

P(o): (PA + NA)/total number of tested concentrations

P(e): {(positive recovery in the absence of food matrix / total number of tested concentrations) x (negative recovery in the absence of food matrix / total number of tested concentrations)} + {(negative recovery in the presence of food matrix / total number of tested concentrations) x (negative recovery in the absence of food matrix / total number of tested concentrations)}.

3. Results and Discussion

3.1. Immuno-magnetic bead combined direct PCR

Several molecular techniques have been developed for the pathogen detection in food samples. However, these techniques involved complex multiple steps that include food matrix separation, pathogen enrichment and sample preparation followed by DNA extraction to avoid possible inhibitions of the downstream amplification (Chapela et al., 2015; Wang and Salazar, 2016). As a consequence, the detection techniques became complex with considerably long duration of analysis (Table S1, *supplementary material*) and limit their practical field applicability. Combination of pathogen concentration with direct PCR is the best efficient approach to avoid these limitations, since the direct PCR bypass the pre-PCR DNA extraction/purification steps (Chin et al., 2017). In this study, a combined approach was adopted to concentrate *Salmonella* Typhimurium cells from food

samples using antibody immobilized magnetic beads and direct PCR was performed without DNA extraction/purification steps (Scheme 1).

3.1.1. Antibody immobilization and inhibitory effect of magnetic beads on the direct PCR method

Strategy to control the immobilization of antibodies on a surface has been the critical parameter in any affinity reactions to retain orientation of antibodies. Immobilization with an appropriate orientation places antibodies in an ideal position to maximize the capacity of antigen recognition (Hermanson, 2013). Protein A/G is known for recognizing the Fc (Fragment crystallizable) domain of an antibody that results in unidirectional orientation of immobilized antibodies. Therefore in this study, protein A/G was used as a bio-adapter to immobilize specifically anti-salmonella antibodies on the magnetic beads. Using this strategy a significant reduction in the absorbance of the antibody-bead conjugate supernatant at 280 nm after overnight incubation was observed and an immobilization efficiency of more than 98% was achieved. The result is in accordance with the manufacturer's claims.

The use of magnetic beads have been proven to be effective in obtaining high sensitivity and selectivity in the pathogen detection (Sun et al., 2015). In an initial experiment it appeared that the uninhibited direct PCR on the magnetic beads needs optimal concentration of Ab-bead conjugate. In order to investigate the possible inhibitory effect of the magnetic bead on the direct PCR, the direct PCR was performed with 0.1 μg, 1 μg, 10 μg and 100 μg of Ab-bead conjugates. This corresponds to 4.8*(10^4, 10^5, 10^6, and 10^7) magnetic bead particles respectively used for the inhibitory tests. The numbers of beads were calculated based on the diameter (1.254 μM) and the density (2 g/cm^3) of the beads (See *supplementary material* for details). The use of magnetic beads showed no inhibitory effect at all on direct PCR even at concentration of 4.8*10^7 beads per 10 μL of PCR reaction (Fig. S2A). In these experiments Ct values from the real-time PCR amplification curves were used as indicators of the inhibitory effects, since significant decreases in PCR efficiency would result in delayed response. A specific amplification of

225 bp of the *hil*A gene (Chin et al., 2017) was obtained that was confirmed in the electrophoresis gel image (Fig. S2B). Analysis of gel image with ImageJ software revealed insignificant differences in the amplified copy numbers (Fig. S2C). This has unlocked the prospect of integrating pathogen concentration and direct amplification of captured *Salmonella* Typhimurium with reduced assay complexity and analysis time.

3.1.2. Pathogen concentration using magnetic beads

Anti-salmonella antibody immobilized magnetic beads tested for direct PCR compatibility was used to capture *Salmonella* Typhimurium cells. 10 μg of Ab-bead conjugate having ~598 ng of immobilized antibody in total was used in the capturing experiments. The protein AG capped magnetic bead used in this study had a binding capacity of 61 μg of antibody per 1 mg of bead, according to the manufacturer. SEM images confirm the capturing of *Salmonella* Typhimurium on the beads (Fig.1). An intact rod shaped *Salmonella* can be seen at higher magnification that was attached to the beads even after multiple washings (Fig. 1C).

3.2. Efficiency and sensitivity of integrated immuno-magnetic direct PCR method

The feasibility of pathogen concentration in combination with direct PCR approach was initially optimized using *Salmonella* Typhimurium spiked PBS. The magnetic bead-based approach had a capturing efficiency of >95%. This was calculated by counting colonies left out in the reaction supernatant after magnetic concentration (Table 1). The capturing efficiencies were also determined based on the ratio of inverse of the slopes obtained in the real-time direct PCR for *Salmonella* Typhimurium before spiking and after recovering *Salmonella* Typhimurium from PBS (Fig. 2A, 2B). According to the slopes the estimated capturing efficiency was around 94% within concentration ranging from 6 to $6.4*10^4$ CFU/mL. This was in agreement with the capturing efficiency calculated by bacterial culture and colony counting method (CFU).

The Direct PCR standard curve generated in the presence of Ab-bead was used to relatively quantify *Salmonella* Typhimurium concentrated from the PBS. For the precise quantification, it is necessary to have similar reaction kinetics and PCR efficiency for samples and the standards (Hedman et al., 2013). In this study, the performance of the

direct PCR was not affected by the presence of magnetic beads. However, it is worth mentioning that there was a delay of approximately 1.5 cycles to reach the threshold fluorescence in the presence of magnetic beads (Fig. S2 A and S2 B). The direct PCR had an efficiency of 87.17% in the presence of magnetic beads and 100.85% in the absence of magnetic beads (Fig. 2A, *Eq. 1*). The difference in the Ct value due to delayed response is an indication of possible inhibitory effects that may results in decreased PCR efficiency (Kralik and Ricchi, 2017). In general, PCR inhibitors are commonly classified as polymerase inhibitors, nucleotide inhibitors and fluorescence inhibitors in particular that interfere with the detection of amplicon during qPCR (Hedman et al., 2013; Schrader et al., 2012). The Pierce protein A/G magnetic beads used in this study had a proprietary double-shell design. The central polymeric core was double-coated with magnetite and sealed with an outer hydrophilic polymer encapsulation surface. This was further covalently coupled with protein A/G monolayer (Protein Biology Application Notes, https://www.thermofisher.com). This may, probably, limit the chances of inhibitions of polymerase and nucleotides. However, this possibility may not be completely ruled out simply because mechanism of PCR inhibition of most of the compounds is not completely known. It was reported that materials with strong background color may interfere with fluorescence detection by simply masking or obstructing the fluorescence (Hedman et al., 2013; Opel et al., 2010). The protein AG magnetic bead had an inherent brownish color. Thus, the difference in the slope of the standard curves may be attributed to the small interference of magnetic beads on the SYBR green fluorescence during the direct PCR (Fig. 2A). In order to confirm this possible effect, total fluorescence of qPCR product (10 μL reaction solution after 35 cycles) with SYBR green was recorded both in the presence and absence of 10 μg of Ab-bead by simply mixing the bead with qPCR product. A reduction (~8-9%) in the SYBR green fluorescence intensity was observed in the presence of 10 μg of Ab-bead conjugate that has confirmed this possibility (Fig. S2 A, Fig. S3 A, B, C). Similarly, a difference observed between the slopes of two standard curves was ~10% (Fig. 2A) that has further supported the possibility. Therefore, the direct PCR standard curve that was generated in the presence of magnetic beads was used to relatively quantify *Salmonella* Typhimurium concentrated from spiked samples (Fig. 2A). Accordingly, pathogen concentration combined direct PCR approach showed convincing detection sensitivity. It was possible to concentrate *Salmonella* Typhimurium at a

concentration as low as 6 CFU/mL from PBS and performing direct PCR on those beads (Fig. S4 and S5). A strong linear relationship (R^2 = 0.9931) was observed between Ct values and the tested concentrations (Fig. 2B). The relative PCR efficiency was ~92% in comparison with standard graph generated in the presence of magnetic bead (Table 1).

3.3. Evaluation of the integrated immuno-magnetic direct PCR method with Salmonella spiked food samples

3.3.1. Effect of food matrix on the direct PCR efficiency and assay sensitivity

The developed integrated immuno-magnetic direct PCR method was used to test different food samples that include vegetable salad, egg white, egg yolk, whole egg and minced pork meat samples. It was possible to detect *Salmonella* Typhimurium within concentration ranging from ~6 to 6.4*10^4 CFU/mL in all the tested food samples except for the egg white (Fig. 3). The relative PCR efficiency was between ~81% and ~94% (Table 1). In the egg white sample, the detection range was between ~65 to 6.4*10^4 CFU/mL with a relative PCR efficiency of 67.3% (Fig. 3D, Table 1). This reduction in the relative PCR efficiency was attributed to the combined effect of capturing efficiency and possible PCR inhibition. The glycoproteins and fats that predominate the composition in the egg white and yolk respectively (Parkinson, 1966) may interfere with sample concentration and PCR. Besides, the food matrix may also affect the pathogen capturing efficiency (Wang et al., 2016). The minced pork meat and the vegetable salad samples are complex matrix and often contains high background of different types of PCR inhibitors, normal microbiota and microflora (Schrader et al., 2012). Monteiro et al. reported that sample dilution can be a simple strategy to reduce the effects of PCR inhibitors (Monteiro et al., 1997). The effect of dilution ratio on the efficiency of direct PCR was also studied in our group previously and a dilution ratio of 1:10 was recommended (Chin et al., 2017). Hence, a dilution ratio of 1:10 was implemented in this study for concentrating *Salmonella* Typhimurium from the food samples. It is worth to mention that lower dilutions were also studied initially; however, the PCR results were inconsistent and were not reproducible (Fig. S6). At a dilution ratio of 1:10 the capturing efficiency was between 85% and 95% (Table 1). This was consistent at lower concentrations of spiked *Salmonella* Typhimurium also as evident from the gel

images (Fig. 3C and insets). Thus, the successful detection of *Salmonella* Typhimurium directly from spiked food samples was attributed to a number of factors. First, the magnetic beads were biocompatible with the Phusion polymerase and did not pose substantial inhibition (Fig. S2). Second, concentrating the spiked pathogen using magnetic beads was efficient in all the tested samples (Table 1). Third, the Phusion polymerase has overcome potential PCR inhibitors of the food samples because of its inherent properties such as enhanced robustness, high accuracy and higher tolerance to inhibitors. The expected positive and negative signals were observed for all assays implying that the sample concentration combined direct PCR approach was suitable for analysis of several food samples without significant interference.

3.3.2. Statistical analysis of the integrated immuno-magnetic direct PCR method

The integrated immuno-magnetic direct PCR assay was statistically evaluated to determine the accuracy, precision and reproducibility of the method. Interpolation of the Ct values of magnetically concentrated sample on the standard graph estimated the recovery from the PBS. Accordingly, an average recovery of 86% was achieved with an average standard deviation (SD) of 0.66 and a median coefficient of variance (CV) of 2.3%. The limit of detection (LoD) and limit of quantification (LoQ) were determined to be ~2 and 2-3 CFU/mL, respectively (*supplementary material,* Table S2).

Further, precision of the integrated immuno-magnetic direct PCR method was determined in the spiked food samples. In comparison with PBS, this integrated method failed to detect *Salmonella* Typhimurium at concentration of 6 CFU/mL in the minced pork meat and in the egg white samples (Fig. 3C). A total number of 60 spiked food samples were tested of which 55 samples were detected positive. This results into a relative accuracy, specificity and sensitivity of 98.3%, 100% and 91.6%, respectively (Table 2). Achieving a relative specificity of 100% was obvious in this assay because of two reasons. First, the antibody used in this study for concentrating *Salmonella* Typhimurium was highly specific to *"O"* (somatic lipopolysaccharide antigen) and *"H"* (flagellar protein) antigens of *Salmonella spp*. Second, the *hil*A gene of *Salmonella* is known to be highly specific for *Salmonella enterica* subspecies Enterica (Cardona-Castro et al., 2002). Chin et al., (2017) has demonstrated the specificity of the *hil*A gene primers to differentiate 15 different

Salmonella serotypes from 16 non-*Salmonella* bacteria strains. This two stage specificity has overcome the possibility of false positive results. The Cohen's kappa index showed excellent agreement (Cohen's kappa = 0.88). In addition, the assay can be completed within 3 hrs with a LoD of ~2 CFU/mL that include sample concentration and specific detection in food samples, which is much better than previously reported studies (Table S1 and S3). This signifies the capability of integrated immuno-magnetic direct PCR method to overcome matrix effects in rapid and ultra-sensitive detection of food borne pathogens.

4. Conclusions

The combination of pathogen concentration and direct PCR strategy was presented in this study as a novel approach for the detection of *Salmonella* Typhimurium directly from food samples without bacterial culturing, DNA isolation and purification steps. The use of magnetic beads in combination with the direct PCR had highly acceptable and reproducible capturing efficiency and PCR amplification efficiency respectively. In contrast to the conventional PCR, the method enabled rapid and ultra-sensitive detection of *Salmonella* Typhimurium at concentrations as low as 2-3 CFU/mL within 3 hrs. The high precision achieved in this integrated immuno-magnetic direct PCR approach with a relative accuracy of 98.3%, a sensitivity of 91.6% and specificity of 100% signifies the potential to overcome the interference from food matrix in the foodborne *Salmonella enterica* detection. This integrated method also possesses potential to be used by the food industries and regulatory agencies for the detection of other pathogens to monitor food quality. The combined approach is also ideally suitable for the integration into a Lab-on-a-chip based biosensor system in future for online monitoring of foodborne pathogens.

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Competing interests

The authors declare no competing financial interest.

Supplementary material

Details of the reagents and the bacterial strain used in this study were provided in the supplementary material. Method followed for the preparation of immuno-magnetic bead conjugate and sample preparation for SEM studies were also provided in the supplementary material.

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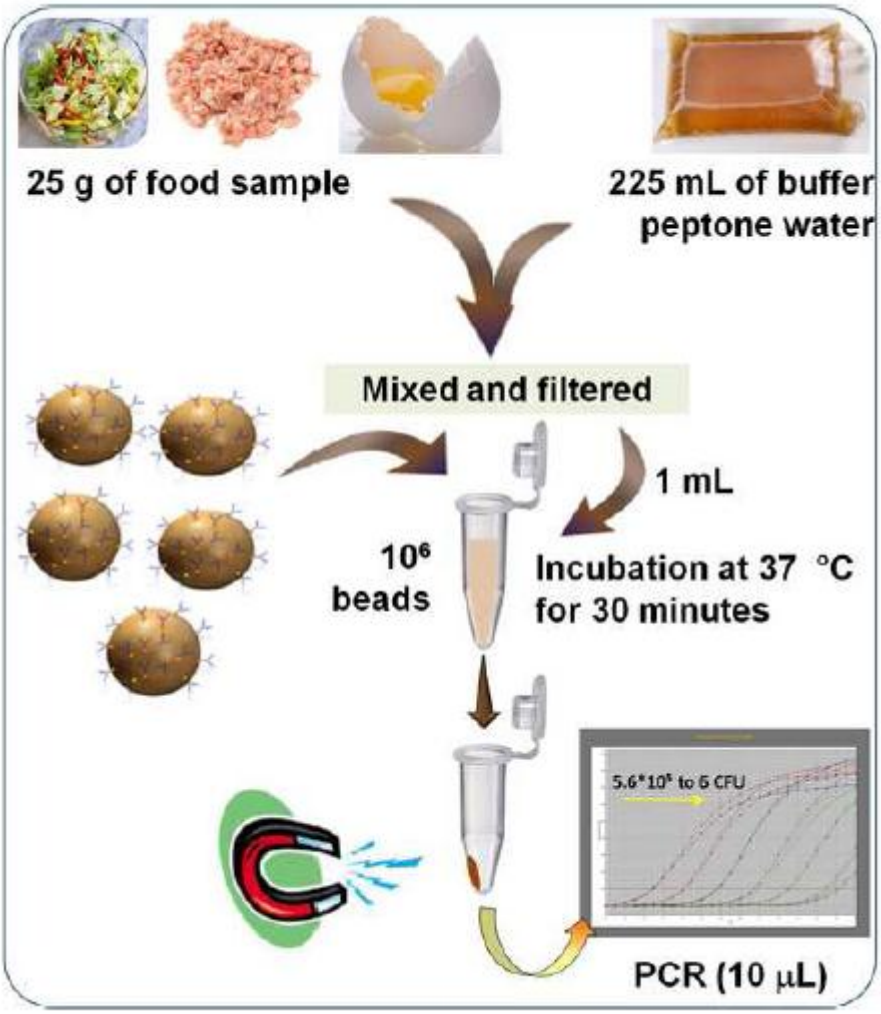
Scheme 1. Schematic representation of the developed integrated immuno-magnetic direct PCR method

Fig. 1. SEM studies on the (A) protein AG capped magnetic beads and (B and C) *Salmonella* Typhimurium-bead complex after capturing.

Fig. 2. Direct PCR standard curves of *Salmonella* Typhimurium (A) before spiking wherein direct PCR was conducted in the absence of magnetic beads (–) and in the presence of magnetic beads (---). (B) Direct PCR linear curve after concentrating *Salmonella* Typhimurium from PBS with magnetic beads. The gel electrophoresis image (both inset A and B) confirmed specific amplification of 225 bp of *hil*A gene respectively. On the gel, Lane 1: negative PCR control without template, lane 2: 5-7 CFU, lane 3: 10^1 CFU, lane 4: 10^2 CFU, lane 5: 10^3 CFU, lane 6: 10^4 CFU, lane 7: 10^5 CFU (for both A and B).

Fig. 3. Direct PCR linear fit curves of *Salmonella* Typhimurium after concentrating with magnetic beads from (A) salad sample, (B) minced pork meat, (D) egg white, (E) egg yolk and (F) whole egg. Dashed line (for all) represent standard curve of *Salmonella* Typhimurium generated in the presence of magnetic beads before spiking. The gel electrophoresis images (all the insets) confirmed specific amplification of 225 bp *hil*A gene in all the tested concentrations. On the gel, lane 1: negative PCR control without template, lane 2: 4-7 CFU, lane 3: 10^1 CFU, lane 4: 10^2 CFU, lane 5: 10^3 CFU, lane 6: 10^4 CFU (for all the insets). (C) Comparison of the capturing efficiency variations between different food samples as revealed by the ImageJ analysis of gel images.



Scheme 1.

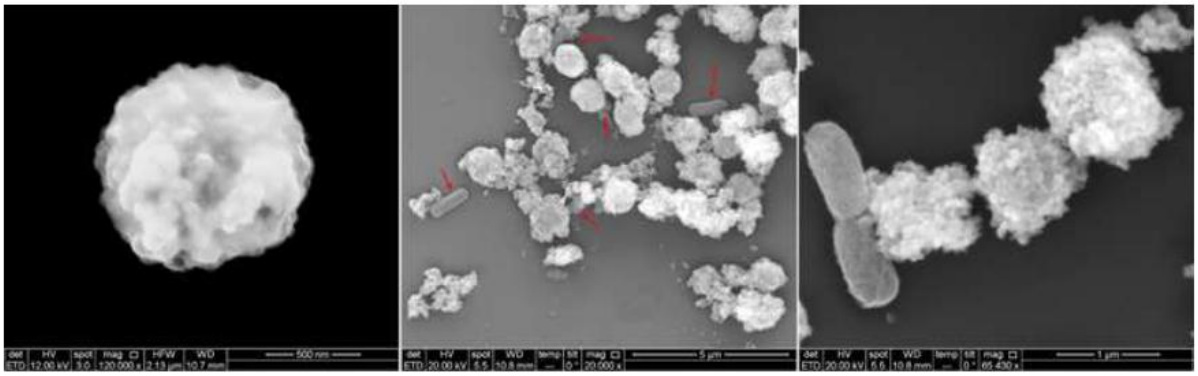


Fig.1.

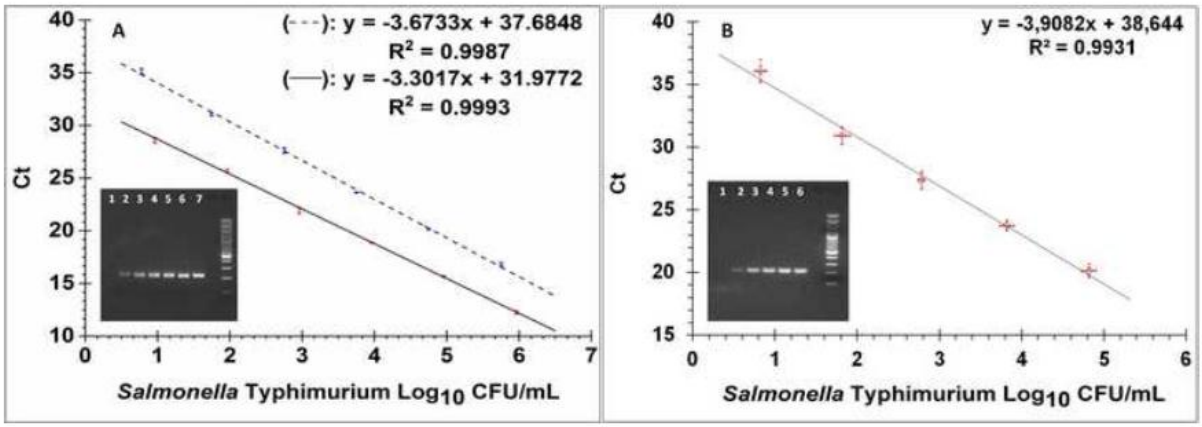

A
(- - -): y = -3.6733x + 37.6848
R² = 0.9987
(—): y = -3.3017x + 31.9772
R² = 0.9993
Ct
Salmonella Typhimurium Log10 CFU/mL
B
y = -3,9082x + 38,644
R² = 0.9931
Ct
Salmonella Typhimurium Log10 CFU/mL

Fig. 2.

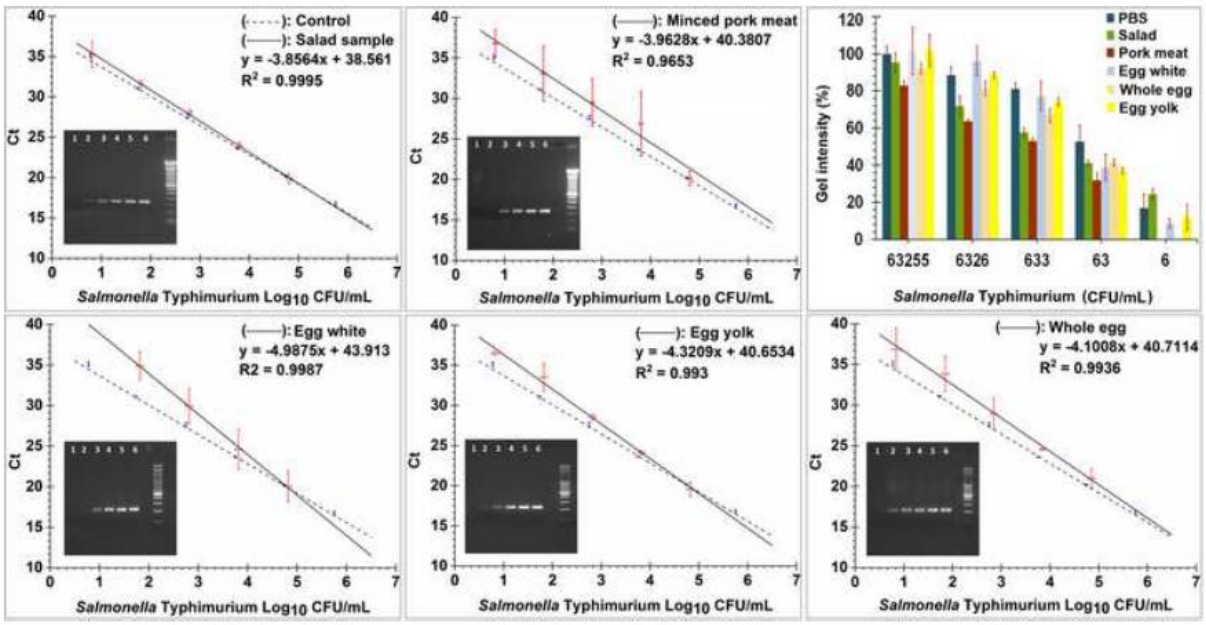


Fig. 3.

Table 1. Comparison of capturing efficiency of anti-salmonella antibody immobilized magnetic bead (based on the XLD plate counts, n=4) and relative PCR efficiencies for *Salmonella* Typhimurium spiked in PBS and different food samples.

	Nature of the sample	Spiked concentration: 1012 CFU/mL		
		Concentration left in the supernatant (CFU/mL)	**[a]Capturing efficiency (%)**	**[b]Relative PCR efficiency (%)**
1.	Standard curve of *Salmonella* Typhimurium with magnetic bead	Not applicable	Not applicable	100
2.	PBS	50	95.12	92.06
3.	Salad sample	85	91.71	93.7
4.	Egg white	80	92.19	67.3
5.	Egg yolk	110	89.27	80.75
6.	Whole egg	50	95.12	86.42
7.	Minced pork meat	145	85.85	90.38

[a]Capturing efficiency was calculated based on the difference of *Salmonella* Typhimurium concentration left in the reaction supernatant after magnetic concentration and initial spiked concentrations.

[b]PCR efficiency was calculated using *Eq 1*. The relative PCR efficiency for different food samples was calculated comparatively considering PCR efficiency of standard curve generated in the presence of magnetic bead as 100%.

Table 2. Assessment of precision of the integrated immuno-magnetic direct PCR method in the spiked food samples. A total of 60 samples were tested of which 55 were reported positive and 5 were false negative. Values were normalized to 100%.

	PBS	Food samples
Average recovery (%)	86	
Test positive (%)	93.3	91.66
Test negative (%)	6.66	8.33
False positive (%)		0
False negative (%)		8.33
Relative accuracy (%)		98.32
Relative specificity (%)		100
Relative sensitivity (%)		91.66
Cohen's kappa index		0.88

Highlights

- An integrated immuno-magnetic direct PCR method to detect *Salmonella* Typhimurium directly from food samples.
- Ultra-sensitive detection without enrichment and DNA extraction steps.
- High precision with a relative accuracy of 98.3% and a sensitivity of 91.6% in the food samples.
- Rapid detection (within 3 hrs) with lowest detection limits (2-3 CFU/mL).