Pathogen Concentration Combined Solid-Phase PCR on Supercritical Angle Fluorescence Microlens Array for Multiplexed Detection of Invasive Nontyphoidal Salmonella Serovars

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Pathogen concentration combined solid phase PCR on supercritical angle fluorescence microlens array for multiplexed detection of invasive non-typhoidal *Salmonella* serovars

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ABSTRACT: Blood stream infections and invasive non-typhoidal *Salmonellosis* in particular remains a major health and economic burden worldwide. The complexity of blood matrices along with extremely low concentration of pathogens in blood pose great challenge for rapid and ultra-sensitive detection. Sample preparation has been the critical step that should provide blood matrix free sample with the targeted pathogen in the highest possible concentration. In this work, we addressed this challenge by combining magnetic bead based pathogen concentration and Solid-phase PCR (SP-PCR). The SP-PCR performed on a super critical angle fluorescence (SAF) microlens array embedded in a microchip enabled quick and accurate detection of low levels of *Salmonella enterica* serovar Typhimurium and Enteritidis in blood samples without culture enrichment. Protein AG-magnetic beads immobilized with anti-salmonella antibody could efficiently concentrate both *Salmonella* serovars with a capturing efficiency >95%. Higher tolerance of Phusion hot start DNA polymerase to PCR inhibitors and its compatibility with protein AG-magnetic beads allowed the integration of SP-PCR. Analysis of *Salmonella* spiked blood samples with the SP-PCR resulted in a limit of detection (LoD) as low as 86 CFU/mL and 94 CFU/mL for *S. Typhimurium* and *S. Enteritidis* respectively that could be attributed to the high fluorescence collection efficiency of the SAF microlens array. These combinations reduced the duration of analysis to less than 3 hours including sample preparation. This platform has the potential for its wide application as a high-throughput biosensor to analyze pathogens in clinical, food and environmental samples.

Bloodstream infections (BSI) caused by a wide variety of pathogens remain a significant burden in both high-income and lower-income countries. BSI accounts for ~0.6 million episodes in North America and about 1.2 million episodes in the European union (EU) resulting in roughly 86000 and 157000 deaths, respectively, every year ¹. Among BSI, invasive non-typhoidal *Salmonella* (iNTS) has gained immense attention as an emerging disease cluster ². *Salmonella* serovar Typhimurium and Enteritidis are identified as the major contributors accounting for an estimated 3.4 million episodes resulting in 0.6 million deaths in sub-Saharan Africa ^{2,3}. The epidemiology and clinical manifestation of iNTS in Asia and South America are poorly described, however, an overall positive prevalence of 27.5% all together in Vietnam, Thailand, Indonesia and 16.2% in Malaysia were reported ^{4,5}. Inability of rapid detection in the early stages of infection remains the major cause of these morbidity and mortality ⁶. Blood culturing, considered as gold standard technique, pose several challenge in the accurate diagnosis of BSI as it has low success rate (30 to 40%) ². It often require more than 72 hours for complete identification of pathogens. In recent times, several multiplex-PCR techniques were reported and made commercially available for whole blood analysis (Table S1). However, these methods are not ideal for rapid point of care applications in high burden and resource-limited settings. Available diagnostic approaches for iNTS either lack clinically relevant sensitivity or have longer analysis time ².

In BSI, the number of recoverable bacteria are often in the range of 1 to 30 CFU/mL that is below the level of analytical sensitivity of many current PCR assays ⁷. Blood matrices being highly complex in nature could adversely influence the assay sensitivity and reproducibility in bioanalytical methods. Therefore, sample preparation has become a critical step in improving blood matrix management and for obtaining better process throughput. In this direction, magnetic separation was reported as an ideal sample concentration method, which is compatible with the downstream molecular analysis ⁸. Magnetic bead based strategy may simultaneously reduce the matrix effects and concentrate the pathogens thereby increasing analytical sensitivity and specificity ⁸. Recently, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been presented as an alternate method to direct molecular analysis of blood samples in BSI (Table S1). However, MALDI-TOF MS is not sensitive enough, thus, requiring long hours of blood culturing and a high abundance of bacteria for proper identification. As an improved approach, an immunoaffinity MALDI-TOF MS in combination with

magnetic bead based sample concentration was reported previously⁹. This integrated MS fingerprinting could detect only 500 cells/ mL in blood serum and 8000 cells/mL in whole blood. In order to address the challenges in direct blood sample analysis, SP-PCR in combination with magnetic bead based sample concentration is presented in this work as a promising strategy (Scheme 1).

In the SP-PCR, pathogen specific probes attached to a surface initiates solid phase amplification. In brief, initial thermal cycling generates amplicons of target genes in the reaction solution (liquid phase) that will binds to the attached pathogen specific probes on the solid surface if the probes match the DNA sequence. In the subsequent thermal cycling, amplification results in initial strand elongation and the extended probes act as templates for the second strand elongation generating new templates for the next round amplification. This result in the PCR products remain covalently attached to the solid support that can be detected by labelling one of the primers with fluorescent dye. In order to attain a superior fluorescence collection efficiency, the SP-PCR was demonstrated (with pure DNA) on a truncated cone-shaped micro-lens array of SAF structures embedded in a polymer microchip^{10–12}. However, the technique was not tested with real sample in the presence of complex matrix such as blood, food and fecal materials. SAF technology is based on the principle of asymmetric distribution of fluorescence near the interface of two media with different refractive indices. This results into emission of majority of the light into the higher refractive index medium. SAF micro-lens arrays exhibit two major advantages such as increased fluorescence collection efficiency and superior multiplexing capacity. In the present work, the SP-PCR was tested with spiked blood samples under multiplexed configuration in a polymer chip with integrated SAF micro-lens arrays. The feasibility of combined effect of magnetic bead based sample concentration and SP-PCR on SAF arrays to overcome challenges in direct blood sample analysis, the effects of blood matrix and rapid analysis were emphasized.

EXPERIMENTAL SECTION

Sample concentration and direct PCR

Salmonella Typhimurium (*S. Typhimurium*) was concentrated from the whole blood using anti-salmonella antibodies immobilized on the protein A/G magnetic beads as described in Supporting Information. Serial 10-fold dilutions of *S. Typhimurium* cells were prepared in 300 μ L of bovine blood stabilized with citrate to attain a final concentrations ranging from 2 CFU to 1.6×10^4 CFU. The spiked

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4 pathogens were captured with 10 μg of Ab-bead conjugate and the beads were separated from the blood.
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6 Further, the volume of the blood was made up to 1 mL with 10 mM PBST and 100 μL of this blood
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8 solution was plated directly on BA plates in duplicates for determining bacterial count. The bacterial
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10 colonies (CFU) were counted after an overnight incubation at 37 $^{\circ}\text{C}$ and the efficiency of Ab-bead to
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12 capture *Salmonella* at different spiking concentrations was calculated.
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14 In order to examine the influence of the whole blood matrix on the direct PCR, various blood dilutions
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16 were prepared by mixing bovine blood with 10 mM PBST to obtain 40% (400 μL blood in 1 mL), 60%
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18 (600 μL blood in 1 mL) and 80% (800 μL blood in 1 mL) blood concentrations. These blood dilutions
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20 and 10 mM PBST (as a negative matrix control) were spiked with 100 μL of *S. Typhimurium* (7.4×10^3
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22 CFU). The spiked *S. Typhimurium* was captured using 10 μg of Ab-bead conjugate and magnetically
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24 concentrated. The beads were washed and direct PCR was performed using Phusion Blood Direct PCR
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26 Kit (Thermo Scientific) as described in Supporting Information. The amplified PCR products were
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28 confirmed by 2% agarose gel electrophoresis.

29 **Effect of blood lysis on the pathogen capturing efficiency and direct PCR**

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31 Blood lysis as an option to improve the pathogen capturing efficiency of Ab-bead conjugate and
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33 performance of the direct PCR was studied. Initially, viability of the *S. Typhimurium* was tested in the
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35 presence of blood lysis agents. Five hundred μL of bovine blood was spiked with 100 μL of *S.*
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37 *Typhimurium* (755 CFU as estimated by culture and colony counting). The spiked blood was mixed with
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39 50 μL of Saponin ^{13,14} to obtain a final concentration of 0.1%, 0.5%, 1%, 2.5% and 5% in separate
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41 experiments and incubated at room temperature for 2 minutes by gentle mixing. Further, 50 μL of
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43 CHAPS ¹⁵ was added to obtain a final concentration of 1%. After incubation for 2 minutes at room
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45 temperature by gentle mixing, volume of the solutions was adjusted to 1 mL with 10 mM PBST. Similar
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47 experiment was conducted in PBS spiked with *S. Typhimurium* (755 CFU) and treated with respective
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49 concentration of Saponin and 1% CHAPS as a control. The samples were incubated at 37 $^{\circ}\text{C}$ for 30
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51 minutes and 100 μL of each reaction solution were plated directly on BA plates in duplicates. Bacterial
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53 colonies (CFU) were determined after an overnight incubation at 37 $^{\circ}\text{C}$ and the viability of *S.*
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55 *Typhimurium* was calculated.
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In order to examine the effect of lysed blood matrix components on the direct PCR, 200 μ L, 400 μ L and 600 μ L of sterile bovine blood was lysed with 2% Saponin and 1% CHAPS as described above. Volume of the lysed blood samples was adjusted to 800 μ L with 10 mM PBST and mixed with 200 μ L of Ab-bead conjugate (10 μ g). The samples were incubated at 37 °C in a bench-top rotator mixer for 30 minutes. The beads were magnetically concentrated and transferred to PCR tubes. An overnight culture of *S. Typhimurium* (3.5×10^4 CFU in 4 μ L) were added to these Ab-bead conjugates concentrated from the lysed sterile blood along with 6 μ L of a direct PCR mixture and direct PCR was performed. The amplified PCR products were confirmed by 2% agarose gel electrophoresis and assessed by ImageJ software (<https://imagej.nih.gov/ij/>) for comparison.

Sensitivity and LoD of the direct PCR in the blood was evaluated by spiking 1 mL of bovine blood with 100 μ L of *S. Typhimurium* from a 10-fold serial dilution prepared from a stock culture ($O.D_{600} = \sim 0.8$) to attain a final concentration gradient from 8 CFU to 8×10^4 CFU/mL of blood. The spiked blood was lysed as described above and the reaction volume was adjusted to 1.8 mL with 10 mM PBST. The spiked pathogens were concentrated with 10 μ g of Ab-bead conjugate (200 μ L) and direct PCR was performed.

Multiplexed SP-PCR

Fabrication and characterization of injection molded SAF microlens array chip. The polymer microchip with SAF microlens array was fabricated by injection molding (Engel Victory 80/45 Tech, PA, USA) as described previously^{10,11}. In brief, a molding insert was initially milled in hard aluminium (alloy 2017, MetalCentret, Denmark) using a computer controlled micro-milling system (Folken Ind., Glendale, California, USA). A 60° engraving tip (DIXI 7006, Le Locle, Switzerland) was used to mill the truncated cone shaped SAF arrays. The metal insert was polished (metal polish, Autosol, USA) to get a smooth surface. Dimension and surface roughness of the SAF structure were measured using scanning electron microscopy (SEM, Quanta 200 FEI) and PLu Neox 3D optical profiler (Sensorfar, USA) respectively. Further, the polished aluminium insert was used to fabricate microchip having 4 reaction chambers (with 32 truncated cone shaped SAF structures in each chamber) in cyclic polyolefin copolymer (COC) (TOPAS 5013-10, Topas Advanced Polymers GmbH, Germany) by injection molding.

Detection of *Salmonella* subtypes with SP-PCR. Multiplexed solid phase amplification was performed in SAF microlens array microchip for detecting *Salmonella* spp. Before SP-PCR, reaction chamber of the microchip containing target specific surface probes immobilized SAF arrays was treated with 25 μ L of 0.1x SSC buffer containing 0.25% bovine serum albumin (BSA) for 30 min at room temperature. Later, the chamber was rinsed with Milli-Q water and dried at room temperature followed by sealing the chamber with thermostable, PCR compatible optical adhesive cover (Applied Biosystems, USA). Ab-bead-*Salmonella* Typhimurium complex and Ab-bead-*Salmonella* Enteritidis complex that were obtained after concentrating spiked *S. Typhimurium* (4.50×10^4 CFU) and *S. Enteritidis* (4.54×10^4 CFU), respectively, from 10 mM PBST in separate experiments were re-suspended back in 10 μ L of 1x Phusion blood PCR buffer. The beads were transferred carefully into separate reaction chambers of the microchip. Later, solid phase amplification was performed with 15 μ L of PCR mixture containing 400 nM of *hlyA* (hyperinvasive locus A) forward and 1600 nM of *hlyA* reverse primers, 200 nM of *sdf* (*Salmonella* difference fragment) forward and 800 nM of *sdf* reverse primers, 600 nM of *fliC* (Flagellin i-antigen) forward and 2400 nM of *fliC* reverse primer (Table S2), 400 ng BSA, 0.05 U/ μ L Phusion blood II DNA polymerase and 1x Phusion blood PCR buffer. The SP-PCR was conducted in a ProFlex™ 2x flat PCR system (Thermo Fisher Scientific). The PCR conditions were 94 °C for 5 min following by 20 cycles of 94 °C for 10s, 60 °C for 20 s, 72 °C for 20 s and another 15 cycles of 94 °C for 10 s, 65 °C for 20 s and 72 °C for 20 s. After the reaction, the chambers were washed with 1x SSC followed by 1x SSC containing 1% Tween-20 for 10 s and 2 times with Milli-Q water for 20 s. The reaction chambers were dried at room temperature before scanning the chip for data analysis.

Analysis of *Salmonella* spiked blood samples. Bovine blood stabilized with citrate (1 mL) was spiked with *S. Typhimurium* and *S. Enteritidis* ($\sim 7 \times 10^4$ CFU of each) in separate experiments and lysed as mentioned above. The spiked pathogens were concentrated using 10 μ g of Ab-bead conjugate after incubating at 37 °C in a bench-top rotator mixer for 30 minutes. The concentrated beads were washed and transferred to reaction chambers in a microchip and SP-PCR was performed as mentioned above. After amplification, the reaction mixture was decanted from the chambers and the amplified PCR products left in the liquid phase were confirmed by 2% agarose gel electrophoresis. The reaction chambers were washed thoroughly as optimized above and dried at room temperature before scanning the chambers for fluorescence imaging.

Sensitivity and LoD of the SP-PCR in the blood was evaluated by spiking 1 mL of anonymous healthy human volunteer blood with *S. Typhimurium* and *S. Enteritidis* in separate experiments. The blood was spiked separately with 100 μ L of *S. Typhimurium* or *S. Enteritidis* from a 10-fold serial dilution prepared from a stock culture ($O.D_{600} = \sim 0.8$ for each) to attain a final concentration gradient from 10^1 CFU/mL to 10^3 CFU/mL of blood. The spiked blood was lysed and the pathogens were concentrated using 10 μ g of Ab-bead conjugate and SP-PCR was performed as described above. After the reaction, reaction mixture (liquid phase) was analyzed by 2% agarose gel electrophoresis while the SAF structures in the microchip (solid phase) was analyzed using microarray scanner to confirm the amplifications.

Image acquisition and analysis. After the solid phase amplification, the microchip was scanned using a BioAnalyzer 4F/4S scanner (LaVision BioTec GmbH, Bielefeld, Germany) with 200-ms exposure time. The captured image was analyzed using ImageJ software¹⁶ and the fluorescence intensity of Cy3 dye was quantified. For precise quantification of fluorescence, area under the SAF structure of the scanned image was restricted with a circle adjusted to the size of the SAF structure. Subsequently, the mean value of grey levels of the pixels inside the circle covering the fluorescent spot was calculated. The mean value of signals of the pixels inside the circle in an area of the microchip without SAF structure was considered as background 1. This background 1 was subtracted from the mean ($n = 3$) signal under the area of the circle on the SAF structure without any immobilized surface probes. This signal was eventually considered as background 2. A signal to noise ratio (SNR) in this study was defined as the mean ($n = 6$) signal intensity of the SAF structures after amplification. The background 2 was subtracted from this mean signal intensity and the resulting value was divided by the standard deviation (SD) of the background 2. The LoD of the assay was estimated at an average SNR of triplicates of lowest bacterial concentration tested¹⁷.

RESULTS AND DISCUSSION

Effect of magnetic beads on the efficiency and sensitivity of direct PCR method

Direct PCR bypass the pre-PCR DNA extraction/purification steps. It opens a way for the integration with pre-pathogen concentration technique that may be the best efficient approach to overcome matrix effects in molecular diagnostics⁸. Hence, a combined approach was adapted, in this study, to concentrate *Salmonella* spp., from spiked blood samples followed by direct PCR. Protein A/G capped magnetic beads

were used to immobilize anti-salmonella antibodies. Affinity of protein A/G for the Fc (Fragment crystallizable) domain of an antibody provides unidirectional orientation to immobilized antibodies. This strategy of immobilization places antibodies in an ideal position for enhanced antigen recognition¹⁸. In our previous study⁸, the effect of magnetic beads on the efficiency and sensitivity of direct PCR method for the detection of *S. Typhimurium* was discussed in detail. It was found in our previous study that protein A/G capped magnetic bead (1.254 μM) enable an antibody immobilization efficiency up to ~98%. Direct PCR conducted in the presence of Ab-bead conjugates resulted in a specific amplification product of 225 bp of the *hlyA* gene. Ab-bead conjugates did not show any inhibitory effect on the direct PCR even at concentration of 4.8×10^7 beads per 10 μL of PCR reaction. Finally, 10 μg of Ab-bead conjugate (4.8×10^6 beads) having ~598 ng of immobilized antibody in total was used in the *Salmonella* capturing experiments. The magnetic bead-based approach had a capturing efficiency of >95% for *S. Typhimurium*. Integrated immuno-magnetic direct PCR method had a relative PCR efficiency of ~92% resulting in a limit of detection (LoD) of ~2 CFU/mL of the spiked food sample⁸.

In the present study, the Ab conjugated beads were used to capture *S. Enteritidis* also and noticed similar capturing efficiency. The antibody used in this study had a polyvalent specificity for *Salmonella* "O" (somatic lipopolysaccharide antigen) and "H" (flagellar protein) antigens of both *S. Typhimurium* and *S. Enteritidis*. This is because the immunogen used to generate antibody was a mixture of *S. Enteritidis*, *S. Typhimurium* and *S. Heidelberg* as specified by the supplier. Therefore, it was possible to concentrate both serovars at a concentration lesser than 10 CFU/mL from PBS. Accordingly, the direct PCR conducted on these pathogen concentrated beads was positive at a concentration as low as 9 CFU/mL and 7 CFU/mL for *S. Typhimurium* and *S. Enteritidis* respectively (Figure1).

Studies on the *Salmonella* spiked blood samples

Effect of blood dilutions on the capturing efficiency. The developed integrated immuno-magnetic direct PCR method was used to test blood samples spiked with *S. Typhimurium*. Initially, citrate stabilized bovine blood was used as matrix to optimize the assay. Ab-bead conjugate could concentrate the *Salmonella* with a capturing efficiency in the range 75-80% as revealed by culture and colony counting method. However, the direct PCR performed on these beads was not successful probably due to PCR inhibition. Repeated washings of the Ab-bead conjugate with PBST after capturing *S.*

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4 Typhimurium did not overcome the effect of inhibitors from the blood. Therefore, dilution of blood as a
5 simple strategy to reduce the effects of PCR inhibitors ¹⁹ was attempted. Dilution of blood had a positive
6 effect on the performance of the direct PCR. The target DNA amplification was better for the samples
7 concentrated at higher blood dilutions as revealed by gel electrophoresis (Figure S1). However, QPCR
8 did not reveal any promising results because of possible quenching of SYBR fluorescence.
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14 **Studies on the blood lysis and direct PCR sensitivity.** Blood lysis was studied as an option to avoid
15 non-specific adsorption of blood cells on the magnetic beads. Hemoglobin, a well-known PCR inhibitor,
16 may interfere with the performance of the direct PCR either by decreasing the polymerase efficiency or
17 by quenching fluorescence of the DNA intercalating dye. Blood cell lysis could provide an ideal
18 condition to washout excess blood matrix and non-specifically adsorbed inhibitors. Researchers have
19 studied Saponin ^{13,14} and CHAPS ¹⁵ as blood cell lysing agents separately. Nevertheless, it is important
20 to consider maintaining viability and intactness of the bacterial cells while lysing blood cells efficiently.
21 It was found, in this study, that 1-2% of Saponin in combination with 1% CHAPS were ideal
22 concentrations to lyse the blood cells completely at which the viability of spiked *Salmonella* spp. was
23 ~90% (Table S3). Gel electrophoresis confirmed the amplification of *hlyA* gene from immuno-
24 magnetically concentrated *S. Typhimurium* as low as 820 CFU in the spiked blood samples (Figure S2).
25 However, SYBR fluorescence remained quenched that made it difficult to identify the true amplification
26 in QPCR at lower concentrations resulting in false negative results (Figure S3). The relative PCR
27 efficiency was ~85% (Figure S4) in the spiked blood samples while it was ~92% in PBS ⁸.
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39 Direct PCR experiments were performed using Phusion blood direct PCR Kit (Thermo Fisher
40 Scientific). The kit is designed to perform PCR directly from whole blood without prior DNA extraction
41 or sample pre-treatment. The Phusion hot start II high-fidelity DNA polymerase used in this kit is a
42 modified polymerase that exhibits extremely high resistance to inhibitors found in blood. High-fidelity,
43 catalytic activity and stability of this enzyme is due to its fusion with a double-strand DNA-binding
44 domain (Sso7d) that improves the performance of the polymerase by guiding negative supercoiling ²⁰.
45 However, the recommended concentration of blood allowed in a PCR reaction using this kit is 5%
46 according to the manufacturer so as to limit the concentrations of inhibitors during PCR. It is important
47 to note that in most of the early infection cases the microbial load can be lesser than 1 CFU/mL in blood.
48 This makes it difficult to directly analyze blood samples without concentration or enrichment of the
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targets⁷. Hence, magnetic bead was used to concentrate *Salmonella* spp. and to get rid of inhibitors from blood matrix. In general, an interference in the QPCR may occur as a result of either polymerase inhibition, nucleotide inhibition or quenching of fluorescence^{21,22}. The Pierce protein A/G magnetic beads do not have any adverse effect on the polymerase activity because of their proprietary double-shell design (Protein Biology Application Notes, <https://www.thermofisher.com>). Their compatibility with direct PCR was studied and reported previously by our group⁸. However, samples with strong background color are known to interfere with fluorescence detection in QPCR by simply masking or obstructing the fluorescence^{22,23}. Inherent brownish color of the protein AG magnetic bead had very low interference on the SYBR green fluorescence during the direct PCR. There was ~8-9% reduction in the SYBR green fluorescence resulting in a delay of approximately 1.5 cycles to reach the threshold fluorescence in the presence of 10 µg of Ab-bead conjugate. This observation was also reported in our previous study⁸. On the other hand, hemoglobin in the blood may act as a strong inhibitor of both polymerase activity and the fluorescence in QPCR. It was reported that whole blood decreases amplification efficiency and cause severe static quenching of DNA intercalating dyes such as SYBR, EvaGreen, ROX and probably others. Binding of free dye molecules in the central cavity of hemoglobin was reported to be a possible mechanism behind these static quenching effects (Sidstedt et al., 2018). This mechanism seems possible as hemoglobin has strong absorption between 500 nm and 600 nm²⁵ that may spectrally overlap with the emission spectra of SYBR dye (522 nm). During magnetic concentration of targets from the blood, the possibility of co-concentration of iron containing hemoglobin along with the beads may not be completely ruled out because the magnetic beads create local magnetic field. Successful amplification as revealed by gel electrophoresis and failed to detect amplicons in QPCR supports this theory (Figure S2 and S3). Thus, inconsistency in the detection of *S. Typhimurium* directly from spiked blood samples was attributed to following factors. First, possible co-concentration of hemoglobin along with *S. Typhimurium* from the lysed blood samples during magnetic concentration; Second, quenching of SYBR fluorescence resulting in failed detection of amplicons; Third, the Phusion polymerase failed to overcome potential PCR inhibitors of the blood samples as the blood matrix in the assay were much beyond the recommended blood volume in the reaction.

Studies on the multiplexed SP-PCR

SP-PCR has become increasingly popular in recent times as an alternate strategy for molecular analysis. Performing SP-PCR on a SAF microlens array structure minimizes undesirable interactions and enables multiplexing capability due to the spatial separation of amplicons. Polymer SAF arrays also increases assay sensitivity because of the principle of collimation of the emitted fluorescent light at supercritical angle thereby enhancing the fluorescence collection efficiency. It was reported that ~1 to 13 fluorophores (per μm^2) can be detected with truncated cone-shaped SAF structure (60° angle that ensure total internal reflection) resulting in 36-40 folds increase in the sensitivity^{10,11}. It is worth mentioning here that fluorophores conjugated to the detector probes are not susceptible to quenchers unlike free DNA intercalating dyes²⁴. Therefore, the SP-PCR on integrated SAF micro-array structures in a lab-on-a-chip platform is an ideal technique to integrate with magnetic bead based sample concentration to overcome inhibition from blood matrix components.

Characterization and application of SAF integrated polymeric chip for *Salmonella* detection.

Multiplexed solid phase amplification was performed in a polymer microchip containing SAF microlens array structures. These microchips were fabricated as reported previously¹¹. Prior to SP-PCR, the microchip was characterized to confirm the fabrication reproducibility and quality of the SAF structures. The reaction chamber in the microchip had a depth of 500 μm with a holding capacity of 23-25 μL reaction volume that contributed to overcome possible bubble formation while loading the samples and enabled proper distribution of the samples in the chip. There were 32 identical SAF structures aligned inside the chamber in a pattern (8×4) at an inter distance of 0.625 μm between the structures (Figure 2a). These SAF structures were ~140 μm wider at the top surface and ~485 μm wider at the bottom with mean surface roughness of ~161 nm (Figure 2b and 2c and Figure S5).

In order to initiate solid phase amplification DNA probes targeting the *hlyA*, *sdf*, and *fliC* genes were directly immobilized on these SAF microlens arrays using UV-induced immobilization technique without any surface modification (Figure 3a). The *hlyA* gene located in *Salmonella* pathogenicity island (SPI-1) is responsible for the regulation of the Type III secretion system (T3SS) in cell invasion. *HlyA* gene being specific for *Salmonella enterica* sub-species Enterica can recognize 57 different *Salmonella* strains in general²⁶. On the other hand, the *fliC* gene targets i-antigen specific phase 1 flagellin in *S.*

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4 Typhimurium^{27,28} and *sdf* (*Salmonella* difference fragment) is specific to *S. Enteritidis*^{29–31}. The SP-
5 PCR conducted on magnetically concentrated *S. Typhimurium* and *S. Enteritidis* in the microchip with
6 SAF structures revealed distinct fluorescence patterns wherein, amplification of *hlyA* gene confirmed
7 *Salmonella* spp. in general and amplification of *fliC* and *sdf* genes differentiated serovar Typhimurium
8 from serovar Enteritidis respectively (Figure 3b and 3c). Amplification of specific combinations of these
9 targeting genes made it possible to detect *Salmonella* at serovar level in a multiplexed configuration.
10 Absence of detectable fluorescence signals from the SAF structures with negative control confirmed the
11 specificity and accuracy of this technique. In the left out reaction liquid, two specific amplicons of 238
12 bp of the *hlyA* gene (between the position 1043 bp and 1281 bp) and 436 bp of the *fliC* gene (between
13 the position 794 bp and 1230 bp) were obtained for *S. Typhimurium*. Similarly, a combination of two
14 specific amplicons of 238 bp of the *hlyA* gene and 311 bp of the *sdf* gene (between the position 20 bp
15 and 331 bp) were obtained for *S. Enteritidis*. Electrophoresis gel image confirmed these specific
16 amplicons as well as the absence of any other amplified products inferring specificity of the primers for
17 the target gene (Figure 3d). In principle, the amplified products of the liquid phase reaction serve as
18 templates for SP-PCR and hence, the results were in accordance with solid phase amplification results.
19 In the SP-PCR, primers were used in different ratios in order to reduce the competition between the
20 unbound primers in the liquid phase and the solid-phase probes during the annealing and extension steps
21 ³². Specificity of *hlyA* gene primers for *Salmonella* genus in general and specificity of *fliC* and *sdf* genes
22 primers for serovar Typhimurium and serovar Enteritidis, respectively, were demonstrated previously by
23 testing 15 different *Salmonella* serotypes and 16 non-*Salmonella* bacteria strains²⁶. In order to improve
24 the amplification efficiency, surface probes with a length of 45 to 80 bps having a short overhang at the
25 5' end were reported as ideal for SP-PCR. The recommended density of such probe for immobilization
26 on the surface was 1.5×10^{11} molecules/mm²³². The length of surface probes used in this study was 60
27 bps. These lengthy probes may interact non-specifically with fluorophore labelled reverse primers as
28 soon as the reaction mixture reaches room temperature after the reaction. On the other hand, fluorophore
29 labelled reverse primers may also adhere to the polymer SAF surfaces as a result of weak hydrophobic
30 interactions. Possibility of interference from traces of blood matrix components co-concentrated along
31 with the magnetic beads may not be neglected. These unintended possibilities may increase the
32 background fluorescence resulting in chances of false positive signals unless stringent post PCR washing
33 steps are not applied. Therefore, a stable washing step was optimized in this study starting sequentially
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with 1x SSC buffer followed by 1x SSC buffer containing 0.1 % Tween-20 and finally with Milli-Q water (Figure S6). As a result, there was a considerable improvement in the S/N ratio (Figure S7).

Ultra-sensitive detection of *Salmonella* subtypes in the spiked blood samples. The magnetic bead integrated SP-PCR method after optimization was used to test volunteer human blood samples spiked with different concentrations of *S. Typhimurium* and *S. Enteritidis*. It was possible to detect *S. Typhimurium* and *S. Enteritidis* at a concentration as low as 86 CFU/mL and 94 CFU/mL, respectively, in the blood (Figure 4a and 4b). The total time of analysis was <3 hrs that includes 5 min for selective blood cell lysis, 40 min for pathogen capturing and magnetic concentration, ~5 min for sample loading in to the ready-to-use chip and sealing, 50 min for SP-PCR, ~5-7 min for chip washing after SP-PCR, 10-15 min for scanning and image acquisition and additional 15 min in total for preparations between the steps. A significant increase in the S/N ration was observed with bacterial concentrations (Figure 4c), although, there were variations in the S/N ratio between the target genes at each concentration.

The variations in the S/N ratio could be attributed to the difference in hybridization efficiencies among targets as the amplicons of liquid phase reaction (Figure 3d) that serve as template for SP-PCR was dissimilar in size. In this study, it was found that SP-PCR was more than 10 fold sensitive than direct PCR combined with magnetic bead based sample concentration in blood samples. The higher sensitivity achieved with SP-PCR was the cumulative effects of magnetic bead based efficient pathogen concentration and sample purification as well as the excellent light collection efficiency of SAF microlens arrays and higher tolerance of solid phase amplification probes to blood matrix inhibitors. These results justify the potential of magnetic bead based sample concentration combined SP-PCR approach for the highly sensitive detection of invasive pathogens with possible multiplexing capabilities without significant interference.

CONCLUSIONS

The combination of pathogen concentration and direct PCR strategy was presented in this study as a novel approach for the detection of *S. Typhimurium* and *S. Enteritidis* directly from spiked blood 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 SP-PCR efficiency. In contrast to the conventional direct PCR, the method enabled rapid and ultra-sensitive detection of *S. Typhimurium*

and *S. Enteritidis* at concentrations as low as 86 CFU/mL and 94 CFU/mL respectively in the blood within 3 hrs. The high precision achieved in this integrated immuno-magnetic SP-PCR approach as a result of excellent light collection efficiency of SAF microlens arrays and higher tolerance of solid phase amplification probes to blood matrix inhibitors signifies the potential to overcome the interference from blood matrix in the detection of pathogens in a multiplexed configuration. 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 rapid monitoring of pathogens in various biological samples without significant interferences.

Supporting Information

Supporting Information contains detailed experimental section, Current techniques available/reported for the detection of pathogens (Table S1), sequences of probes and primers for bacterial detection (Table S2), studies on the effects of blood lysing agents on the bacterial viability (Table S3), and supporting figures.

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Notes

All authors have given approval to the final version of the manuscript. The authors declare no competing financial interest.

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Figures legend

Scheme 1. Schematic representation of the magnetic bead based sample concentration combined SP-PCR. (A) Selective blood cell lysis, (A1) Infected blood before lysis, (A2) Infected blood after lysis, (B) Antibody immobilized protein AG magnetic bead, (C) Magnetically captured *Salmonella* cell, (D) SP-PCR and optical detection, (D1) SAF embedded microchip, (D2) SAF micro-lens array, (D3) Target specific amplification on SAF structures, (D4) Image capture after amplification

Figure 1. Direct PCR linear curve after concentrating *Salmonella* Typhimurium and *Salmonella* Enteritidis from PBS with magnetic beads.

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Figure 2. SEM image of the SAF micro-array and polymer microchip. (a) SAF arrays, (b) Truncated cone shaped SAF structure, (c) Polymer microchip with 4 reaction chambers.

Figure 3. Multiplexed detection of Typhimurium and Enteritidis concentrated from PBS using magnetic beads. (a) Pattern of spotting of target probe on the SAF arrays, (b) SP-PCR of Enteritidis, (c) SP-PCR of Typhimurium, (d) Gel electrophoresis image of amplicons in the leftover liquid from SP-PCR reaction, lane 1: Molecular marker, lane 2 and 3: Negative controls, lane 4 and 5: *S. Enteritidis*, lane 6 and 7: *S. Typhimurium*.

Figure 4. SP-PCR for multiplexed detection of Typhimurium and Enteritidis concentrated from spiked blood using magnetic beads. (A) Typhimurium, (B) Enteritidis, (C) Comparison of SNR and sensitivity of the SP-PCR amplification of pathogen specific target genes performed on the SAF microlens array in multiplexed configuration.

Scheme 1. Schematic representation of the magnetic bead based sample concentration combined SP-PCR. (A) Selective blood cell lysis, (A1) Infected blood before lysis, (A2) Infected blood after lysis, (B) Antibody immobilized protein AG magnetic bead, (C) Magnetically captured *Salmonella* cell, (D) SP-PCR and optical detection, (D1) SAF embedded microchip, (D2) SAF micro-lens array, (D3) Target specific amplification on SAF structures, (D4) Image capture after amplification

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Figure 1. Direct PCR linear curve of *S. Typhimurium* and *S. Enteritidis* after concentrating from PBS with magnetic beads.

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Figure 2. SEM image of the SAF micro-array and polymer microchip. (a) SAF arrays, (b) Truncated cone shaped SAF structure, (c) Polymer microchip with 4 reaction chambers.

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Figure 3. Multiplexed detection of Typhimurium and Enteritidis concentrated from PBS using magnetic beads. (a) Pattern of spotting of target probe on the SAF arrays, (b) SP-PCR of Enteritidis, (c) SP-PCR of Typhimurium, (d) Gel electrophoresis image of amplicons in the leftover liquid from SP-PCR reaction, lane 1: Molecular marker, lane 2 and 3: Negative controls, lane 4 and 5: S. Enteritidis, lane 6 and 7: S. Typhimurium.

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Figure 4. SP-PCR for multiplexed detection of Typhimurium and Enteritidis concentrated from spiked blood using magnetic beads. (A) Typhimurium, (B) Enteritidis, (C) Comparison of SNR and sensitivity of the SP-PCR amplification of pathogen specific target genes performed on the SAF microlens array in multiplexed configuration.

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