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

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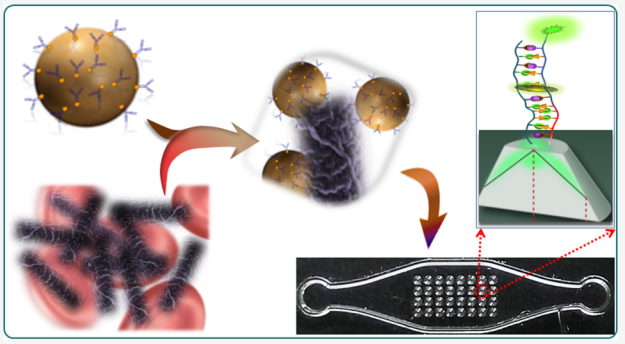
ABSTRACT: Bloodstream infections and invasive nontyphoidal *Salmonellosis* in particular remain a major health and economic burden worldwide. The complexity of blood matrixes along with extremely low concentration of pathogens in blood poses a great challenge for rapid and ultrasensitive detection. Sample 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 supercritical 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 antisalmonella 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 h including sample preparation. This platform has the potential for wide application as a high-throughput biosensor to analyze pathogens in clinical, food, and environmental samples.



Bloodstream infections (BSIs) caused by a wide variety of pathogens remain a significant burden in both high-income and lower-income countries. BSIs account 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 BSIs, invasive nontyphoidal *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, and Indonesia and 16.2% in Malaysia was reported.[4,5] Inability of rapid detection in the early stages of infection remains the major cause of the morbidity and mortality.[6] Blood culturing, considered as the gold standard technique, poses several challenges in the accurate diagnosis of BSI, as it has low success rate (30−40%).[2] It often requires more than 72 h 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 matrixes, being highly complex in nature, can 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

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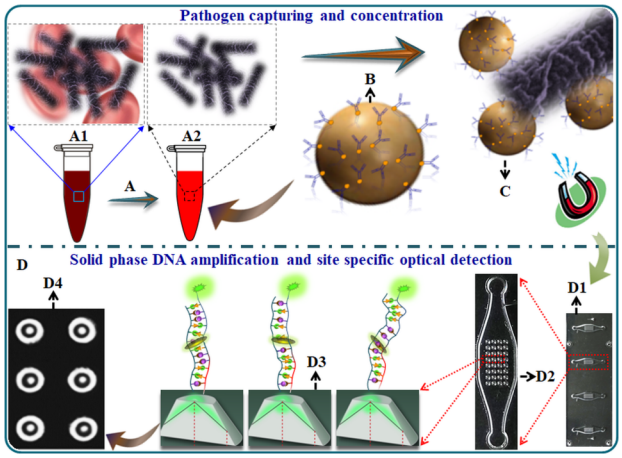
matrix effects and concentrate the pathogens thereby increasing analytical sensitivity and specificity.[8] Recently, matrix-assisted laser desorption/ionization time-of-fight mass spectrometry (MALDI-TOF MS) has been presented as an alternate method to direct molecular analysis of blood samples in BSI (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 previously.[9] This integrated MS fingerprinting could detect only 500 cells/mL in blood serum and 8000 cells/mL in whole blood. 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).

Scheme 1. Magnetic-Bead-Based Sample Concentration Combined SP-PCR[a]



[a](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 microlens array, (D3) target-specific amplification on SAF structures, (D4) image capture after amplification.

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 bind 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 results in the PCR products remaining covalently attached to the solid support that can be detected by labeling one of the primers with fluorescent dye. To attain a superior fluorescence collection efficiency, the SP-PCR was demonstrated (with pure DNA) on a truncated cone-shaped microlens array of SAF structures embedded in a polymer microchip.[10−12] However, the technique was not tested with real samples in the presence of a 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 in the emission of the majority of light into the higher refractive index medium. SAF microlens 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 microlens arrays. The feasibility of the 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 was emphasized.

EXPERIMENTAL SECTION

Sample Concentration and Direct PCR. *Salmonella typhimurium* (*S. typhimurium*) was concentrated from whole blood using antisalmonella antibodies immobilized on protein A/G magnetic beads as described in Supporting Information. Serial 10-fold dilutions of *S. typhimurium* cells were prepared in 300 μL of bovine blood stabilized with citrate to attain final concentrations ranging from 2 CFU to 1.6×10^4 CFU. The spiked pathogens were captured with 10 μg of Ab−bead conjugate, and the beads were separated from the blood. Further, the volume of blood was made up to 1 mL with 10 mM PBST, and 100 μL of this blood solution was plated directly on BA plates in duplicates for determining bacterial count. The bacterial colonies (CFU) were counted after an overnight incubation at 37 °C, and the efficiency of Ab−beads to capture *Salmonella* at different spiking concentrations was calculated.

To examine the influence of the whole blood matrix on the direct PCR, various blood dilutions were prepared by mixing bovine blood with 10 mM PBST to obtain 40% (400 μL of blood in 1 mL), 60% (600 μL of blood in 1 mL), and 80% (800 μL of blood in 1 mL) blood concentrations. These blood dilutions and 10 mM PBST (as a negative matrix control) were spiked with 100 μL of *S. typhimurium* (7.4×10^3 CFU). The spiked *S. typhimurium* was captured using 10 μg of Ab−bead conjugate and magnetically concentrated. The beads were washed, and direct PCR was performed using Phusion Blood Direct PCR Kit (Thermo Scientific) as described in Supporting Information. The amplified PCR products were confirmed by 2% agarose gel electrophoresis.

Effect of Blood Lysis on the Pathogen Capturing Efficiency and Direct PCR. Blood lysis as an option to improve the pathogen capturing efficiency of Ab−bead conjugate and performance of the direct PCR were studied. Initially, viability of *S. typhimurium* was tested in the presence of blood lysis agents. Five hundred microliters of bovine blood was spiked with 100 μL of *S. typhimurium* (755 CFU as estimated by culture and colony counting). The spiked blood was mixed with 50 μL of saponin[13,14] to obtain a final concentration of 0.1%, 0.5%, 1%, 2.5%, and 5% in separate experiments and incubated at room temperature for 2 min by gentle mixing. Further, 50 μL of CHAPS[15] was added to obtain a final concentration of 1%. After incubation for 2 min at room temperature by gentle mixing, the volume of the solutions was adjusted to 1 mL with 10 mM PBST. A similar experiment was conducted in PBS spiked with *S. typhimurium* (755 CFU) and treated with respective concentration of saponin and 1% CHAPS as a control. The samples were incubated at 37 °C for 30 min, and 100 μL of each reaction solution was plated directly on BA plates in duplicates. Bacterial colonies (CFU)

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were determined after an overnight incubation at 37 °C, and the viability of *S. typhimurium* was calculated.

To examine the effect of lysed blood matrix components on the direct PCR, 200, 400, and 600 μL of sterile bovine blood were lysed with 2% saponin and 1% CHAPS as described above. The volume of the lysed blood samples was adjusted to 800 μL with 10 mM PBST and mixed with 200 μL of Ab−bead conjugate (10 μg). The samples were incubated at 37 °C in a benchtop rotator mixer for 30 min. The beads were magnetically concentrated and transferred to PCR tubes. An overnight culture of *S. typhimurium* (3.5×10^4 CFU in 4 μL) was added to these Ab−bead conjugates concentrated from the lysed sterile blood along with 6 μL 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 were evaluated by spiking 1 mL of bovine blood with 100 μL of *S. typhimurium* from a 10-fold serial dilution prepared from a stock culture (OD_{600} = ∼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 μg of Ab−bead conjugate (200 μL), 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, York, PA) as described previously.[10,11] In brief, a molding insert was initially milled in hard aluminum (alloy 2017, MetalCentret, Denmark) using a computer-controlled micromilling system (Folken Ind., Glendale, CA). 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, Austin, TX) 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, Newington, CT) respectively. Further, the polished aluminum insert was used to fabricate microchip having four 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 the SAF microlens array microchip for detecting *Salmonella* spp. Before SP-PCR, the reaction chamber of the microchip containing target-specific surface-probe-immobilized SAF arrays was treated with 25 μL of 0.1X SSC buffer containing 0.25% bovine serum albumin (BSA) for 30 min at room temperature. Later, the chamber was rinsed with Millli-Q water and dried at room temperature followed by sealing the chamber with thermostable, PCR-compatible optical adhesive cover (Applied Biosystems, Waltham, MA). 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 resuspended back in 10 μL 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 μL of PCR mixture containing 400 nM of *hil*A (hyperinvasive locus A) forward and 1600 nM of *hil*A reverse primers, 200 nM of *sdf* (*Salmonella* difference fragment) forward and 800 nM of *sdf* reverse primers, 600 nM of *fli*C (flagellin i-antigen) forward and 2400 nM of *fli*C reverse primer (Table S2), 400 ng of BSA, 0.05 U/μL Phusion blood II DNA polymerase and 1X Phusion blood PCR buffer. The SP-PCR was conducted in a ProFlexTM 2X flat PCR system (Thermo Fisher Scientific). The PCR conditions were 94 °C for 5 min followed by 20 cycles of 94 °C for 10 s, 60 °C for 20 s, and 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 two 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* (∼7×10^4 CFU of each) in separate experiments and lysed as mentioned above. The spiked pathogens were concentrated using 10 μg of Ab−bead conjugate after incubating at 37 °C in a benchtop rotator mixer for 30 min. 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 μL of *S. typhimurium* or *S. enteritidis* from a 10-fold serial dilution prepared from a stock culture (OD_{600} = ∼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 μg of Ab−bead conjugate, and SP-PCR was performed as described above. After the reaction, the reaction mixture (liquid phase) was analyzed by 2% agarose gel electrophoresis while the SAF structures in the microchip (solid phase) were analyzed using a 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 software,[16] 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 gray 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 the 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

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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 the lowest bacterial concentration tested.[17]

RESULTS AND DISCUSSION

Effect of Magnetic Beads on the Efficiency and Sensitivity of Direct PCR Method. Direct PCR bypasses the pre-PCR DNA extraction/purification steps. It opens a way for integration with the pathogen concentration technique that may be the best efficient approach to overcome matrix effects in molecular diagnostics.[8] 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 antisalmonella 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 recognition.[18] In our previous study,[8] the effect of magnetic beads on the efficiency and sensitivity of the 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 beads (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 *hil*A gene. Ab−bead conjugates did not show any inhibitory effect on the direct PCR even at a 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*. The integrated immunomagnetic 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 sample.[8]

In the present study, the Ab-conjugated beads were used to capture *S. enteritidis* also, and a similar capturing efficiency was noticed. 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. enteriditis*, *S. typhimurium*, and *S. heidelburg* as specified by the supplier. Therefore, it was possible to concentrate both serovars at a concentration less 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 (Figure 1).

Studies on the *Salmonella*-Spiked Blood Samples. *Effect of Blood Dilutions on the Capturing Efficiency.* The developed integrated immunomagnetic direct PCR method was used to test blood samples spiked with *S. typhimurium*. Initially, citrate-stabilized bovine blood was used as a matrix to optimize the assay. Ab−bead conjugate could concentrate the *Salmonella* with a capturing efficiency in the range 75−80% as revealed by the 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. typhimurium*

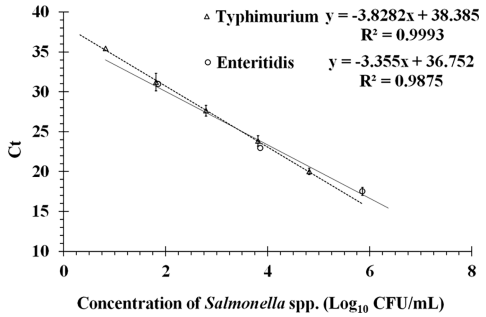

Figure 1. Direct PCR linear curve after concentrating *S. typhimurium* and *S. enteritidis* from PBS with magnetic beads.

did not overcome the effect of inhibitors from the blood. Therefore, dilution of blood as a simple strategy to reduce the effects of PCR inhibitors[19] was attempted. Dilution of blood had a positive effect on the performance of the direct PCR. The target DNA amplification was better for the samples concentrated at higher blood dilutions as revealed by gel electrophoresis (Figure S1). However, QPCR did not reveal any promising results because of possible quenching of SYBR fluorescence.

Studies on Blood Lysis and Direct PCR Sensitivity. Blood lysis was studied as an option to avoid nonspecific adsorption of blood cells on the magnetic beads. Hemoglobin, a well-known PCR inhibitor, may interfere with the performance of the direct PCR either by decreasing the polymerase efficiency or by quenching fluorescence of the DNA intercalating dye. Blood cell lysis could provide an ideal condition to washout excess blood matrix and nonspecifically adsorbed inhibitors. Researchers have studied saponin[13,14] and CHAPS[15] as blood cell lysing agents separately. Nevertheless, it is important to consider maintaining viability and intactness of the bacterial cells while lysing blood cells efficiently. It was found, in this study, that 1−2% of saponin in combination with 1% CHAPS were ideal concentrations to lyse the blood cells completely at which the viability of spiked *Salmonella* spp. was ~90% (Table S3). Gel electrophoresis confirmed the amplification of *hil*A gene from immunomagnetically concentrated *S. typhimurium* as low as 820 CFU in the spiked blood samples (Figure S2). However, SYBR fluorescence remained quenched that made it difficult to identify the true amplification in QPCR at lower concentrations, resulting in false negative results (Figure S3). The relative PCR efficiency was ~85% (Figure S4) in the spiked blood samples while it was ~92% in PBS.[8]

Direct PCR experiments were performed using Phusion blood direct PCR Kit (Thermo Fisher Scientific). The kit is designed to perform PCR directly from whole blood without prior DNA extraction or sample pretreatment. The Phusion hot start II high-fidelity DNA polymerase used in this kit is a modified polymerase that exhibits extremely high resistance to inhibitors found in blood. High fidelity, catalytic activity, and stability of this enzyme are due to its fusion with a double-strand DNA-binding domain (Sso7d) that improves the performance of the polymerase by guiding negative super-coiling.[20] However, the recommended concentration of blood allowed in a PCR reaction using this kit is 5% according to the manufacturer so as to limit the concentrations of inhibitors during PCR. It is important to note that in most of the early infection cases the microbial load can be less than 1 CFU/mL in blood. This makes it difficult to directly analyze blood samples without concentration or enrichment of the targets.[7]

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Hence, magnetic beads were used to concentrate *Salmonella* spp. and to eliminate inhibitors from the blood matrix. In general, 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 group.[8] 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 beads 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 study.[8] 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 causes 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.[24] This mechanism seems possible, as hemoglobin has strong absorption between 500 and 600 nm[25] that may spectrally overlap with the emission spectra of SYBR dye (522 nm). During magnetic concentration of targets from the blood, the possibility of concentration of iron-containing hemoglobin along with the beads may not be completely ruled out because the magnetic beads create a local magnetic field. Successful amplification as revealed by gel electrophoresis and absence of detectable fluorescence signals in QPCR supports this theory (Figures S2 and S3). Thus, inconsistency in the detection of *S. typhimurium* directly from spiked blood samples was attributed to the following factors. First, possible 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 was 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 an SAF microlens array structure minimizes undesirable interactions and enables multiplexing capability due to the spatial separation of amplicons. Polymer SAF arrays also increase assay sensitivity because of the principle of collimation of the emitted fluorescent light at a 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 ensures total internal reflection), resulting in a 36−40 fold 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 dyes.[24] Therefore, the SP-PCR on integrated SAF microarray structures in a lab-on-a-chip platform is an ideal technique to integrate with a 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 previously.[11] 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 interdistance 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 a mean surface roughness of ∼161 nm (Figure 2b,c and Figure S5).

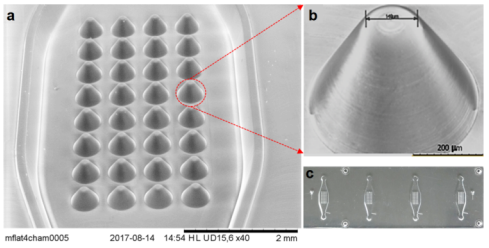

Figure 2. SEM image of the SAF microarray and polymer microchip. (a) SAF arrays. (b) Truncated cone-shaped SAF structure. (c) Polymer microchip with four reaction chambers.

To initiate solid-phase amplification, DNA probes targeting the *hil*A, *sdf*, and *fli*C genes were directly immobilized on the SAF microlens arrays using UV-induced immobilization without any surface modification (Figure 3a). The *hil*A gene

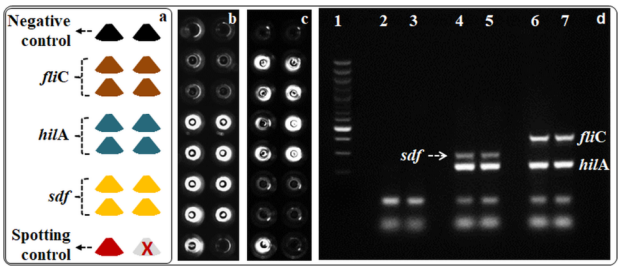

Figure 3. Multiplexed detection of *S. typhimurium* and *S. enteritidis* concentrated from PBS using magnetic beads. (a) Pattern of spotting of target probe on the SAF arrays. (b) SP-PCR of *S. enteritidis*. (c) SP-PCR of *S. typhimurium*. (d) Gel electrophoresis image of amplicons in the leftover liquid from SP-PCR reaction. Lane 1: molecular marker; lanes 2 and 3: negative controls; lanes 4 and 5: *S. enteritidis*; lanes 6 and 7: *S. typhimurium*.

located in the *Salmonella* pathogenicity island (SPI-1) is responsible for the regulation of the type III secretion system (T3SS) in cell invasion. The *hil*A gene, being specific for *Salmonella enterica* subspecies *enterica*, can recognize 57 different *Salmonella* strains in general.[26] On the other hand, the *fli*C gene targets i-antigen-specific phase 1 flagellin in *S. typhimurium*,[27,28] and *sdf* (*Salmonella* difference fragment) is specific to *S. enteritidis*.[29−31] The SP-PCR conducted on

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magnetically concentrated *S. typhimurium* and *S. enteritidis* in the microchip with SAF structures revealed distinct fluorescence patterns wherein amplification of the *hil*A gene confirmed *Salmonella* spp. in general, and amplification of *fli*C and *sdf* genes differentiated serovar *typhimurium* from serovar *enteritidis*, respectively (Figure 3b,c). Amplification of specific combinations of these targeting genes made it possible to detect *Salmonella* at serovar level in a multiplexed configuration. Absence of detectable fluorescence signals from the SAF structures with negative control confirmed the specificity and accuracy of this technique. In the left-out reaction liquid, two specific amplicons of 238 bp of the *hil*A gene (between the positions 1043 bp and 1281 bp) and 436 bp of the *fli*C gene (between the positions 794 bp and 1230 bp) were obtained for *S. typhimurium*. Similarly, a combination of two specific amplicons of 238 bp of the *hil*A gene and 311 bp of the *sdf* gene (between the positions 20 bp and 331 bp) was obtained for *S. enteritidis*. The electrophoresis gel image confirmed these specific amplicons as well as the absence of any other amplified products inferring specificity of the primers for the target gene (Figure 3d). In principle, the amplified products of the liquid-phase reaction serve as templates for SP-PCR, and hence the results were in accordance with solid-phase amplification results. In the SP-PCR, primers were used in different ratios to reduce the competition between the unbound primers in the liquid phase and the solid-phase probes during the annealing and extension steps.[32] Specificity of *hil*A gene primers for *Salmonella* genus in general and specificity of *fli*C and *sdf* genes primers for serovar *typhimurium* and serovar *enteritidis*, respectively, were demonstrated previously by testing 15 different *Salmonella* serotypes and 16 non-*Salmonella* bacteria strains.[26] To improve the amplification efficiency, surface probes with a length of 45 to 80 bps having a short overhang at the 5′ end were reported as ideal for SP-PCR. The recommended density of such probes for immobilization on the surface was 1.5×10^{11} molecules/mm^2.[32] The length of surface probes used in this study was 60 bp. These lengthy probes may interact nonspecifically with fluorophore-labeled reverse primers as soon as the reaction mixture reaches room temperature after the reaction. On the other hand, fluorophore-labeled reverse primers may also adhere to the polymer SAF surfaces as a result of weak hydrophobic interactions. Possibility of interference from traces of blood matrix components concentrated along with the magnetic beads may not be neglected. These unintended possibilities may increase the background fluorescence, resulting in chances of false positive signals unless stringent post-PCR washing steps are applied. Therefore, a stable washing step was optimized in this study starting sequentially 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).

Ultrasensitive 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,b). The total time of analysis was <3 h that includes 5 min for selective blood cell lysis, 40 min for pathogen capture and magnetic concentration, ∼5 min for sample loading into the ready-to-use chip and sealing, 50 min for SP-PCR, ∼5−7 min

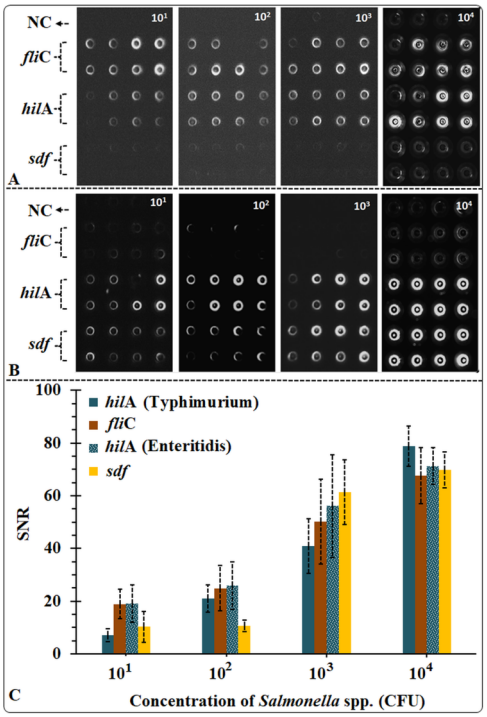

Figure 4. SP-PCR for multiplexed detection of *S. typhimurium* and *S. enteritidis* concentrated from spiked blood using magnetic beads: (A) *S. typhimurium*, (B) *S. enteritidis*. (C) Comparison of SNR and sensitivity of the SP-PCR amplification of pathogen-specific target genes performed on the SAF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

for chip washing after SP-PCR, 10−15 min for scanning and image acquisition, and an additional 15 min in total for preparations between the steps. A significant increase in the S/N ratio 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 the liquid-phase reaction (Figure 3d) that serve as a template for SP-PCR were dissimilar in size. In this study, it was found that SP-PCR was more than 10-fold more sensitive than direct PCR combined with magnetic-bead-based sample concentration in blood samples. The higher sensitivity achieved with SP-PCR was due to 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 the 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 conventional

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direct PCR, the method enabled rapid and ultrasensitive 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 h. The high precision achieved in this integrated immunomagnetic 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 the 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 the future for rapid monitoring of pathogens in various biological samples without significant interference.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.9b04863.

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 (PDF)

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Notes

The authors declare no competing financial interest.

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