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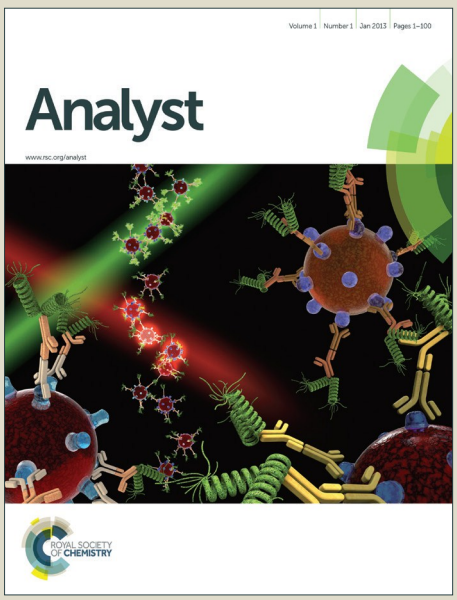

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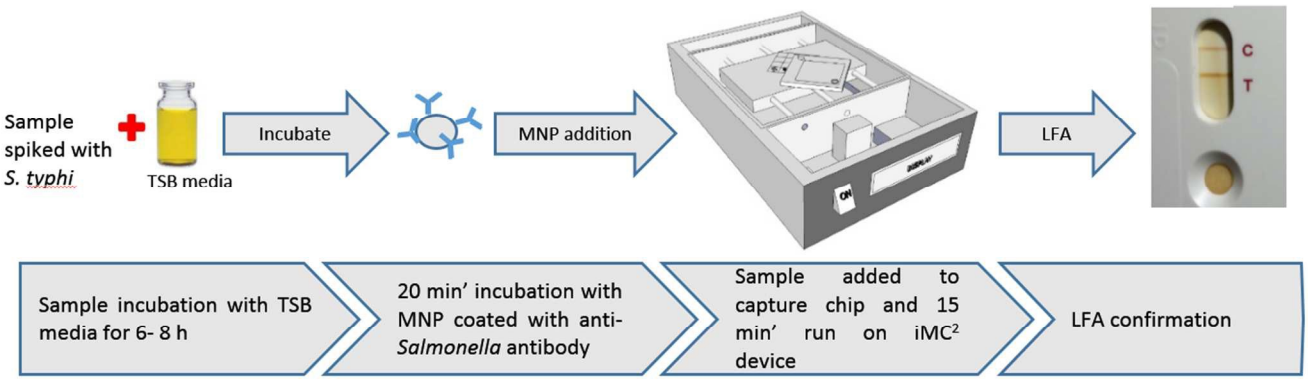

An affordable immuno-magnetic cell capture system for bacterial detection in 7 hours with 10 CFU/ml sensitivity.

A portable immunomagnetic cell capture system to accelerate culture diagnosis of bacterial infections

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Abstract:

Bacterial infections continue to be a major cause of deaths globally, particularly in resource-poor settings. In the absence of rapid and affordable diagnostic solutions, patients are mostly administered broad spectrum antibiotics leading to antibiotics resistance and poor recovery. Culture diagnosis continues to be gold standard for diagnosis of bacterial infection, despite its long turnaround time of 24 to 48 h. We have developed a portable immunomagnetic cell capture (iMC^2) system that allows rapid culture diagnosis of bacterial pathogens. Our approach involves culture growth of the blood samples in broth media for 6 to 8 h, followed by immunomagnetic enrichment of the target cells using the iMC^2 device. The device comprises of a disposable capture chip that has two chambers of 5 ml and 50 µl volume connected through a channel with a manual valve. Bacterial cells bound to antibody coated magnetic nanoparticles are swept from the 5 ml sample chamber into the 50 µl recovery chamber by moving an external magnetic field with respect to the capture chip using a linear positioner. This enables specific isolation and up to 100× enrichment of the target cells. The presence of bacteria in the recovered sample is confirmed visually using a lateral flow immunoassay. The system is demonstrated in buffer and blood samples spiked with *S.typhi*. The method has high sensitivity (10 CFU/ml), specificity and a rapid turnaround time of less than 7 h, a significant improvement over conventional methods.

INTRODUCTION

Despite continuous and rapid advancements made in medical healthcare technologies, bloodstream bacterial infections continue to pose a great threat to the society[1]. While antibiotic therapies are generally effective in combating such infections, lack of timely diagnosis is linked to the high rates of morbidity and mortality, and increased costs of treatment[2]. In the absence of

rapid and affordable diagnostics solutions, the clinicians are often forced to start the patients on a broad-spectrum antibiotic regimen before getting a full diagnostic confirmation of their disease. Such rampant use of antibiotics has led to the development of drug resistance among microorganisms and increased the complexity of treatment procedures[2-4].

Blood culture is considered a 'gold standard' method to investigate identify bacterial pathogens in blood even though it can take 24 to 48 h for preliminary confirmation of positive culture and microbe identification tests [5, 6]. The standard blood culture process can be subdivided into two stages: a pre-analytical and an analytical stage. The pre-analytical stage consists of all the steps involved from sample collection to its loading into the bacterial culture broth. The time taken during this stage can be drastically reduced by use of point-of-care systems that can be deployed directly at the primary healthcare site. The analytical phase involves the culture growth of microbes to a threshold concentration followed by pathogen identification. Several fully-automated and continuous-monitoring blood culture systems have been developed and are commercially available[7, 8]. These systems are optimized on the basis of the use of media [2, 9], antimicrobial neutralization[10, 11], incubation time[12], temperature, agitation[13] *etc.* to achieve a faster confirmation of positive culture. After positive flagging of bacterial growth, a definitive confirmation of the species is done using detection methods such as microscopy, bead agglutination tests, enzyme linked immunosorbent assays (ELISA), tube coagulase, PCR based assay and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS)[5, 14-17]. A very important and crucial limitation of these systems, however, is the large turnaround time taken for definitive pathogen identification[18]. Furthermore, these systems are not suitable for resource-poor settings due to their complexity of operation and unaffordable cost.

The time taken for blood culture diagnosis of a specific pathogen can be reduced considerably by incorporating an enrichment protocol. In this regard, immunomagnetic separation stands out as a facile enrichment method to isolate specific pathogens from complex biological fluids such as urine, blood, stool, cerebrospinal fluid *etc*. [19-21] into a smaller volume and to remove background molecules that can potentially interfere with the downstream signal detection.

In a typical immunomagnetic capture setup, the sample of interest is incubated with excess magnetic nanoparticles (MNPs) pre-conjugated with antibodies specific for a particular target [22]. DNA aptamers, lectins, peptides etc. may also be used in place of the antibody as long as the ligand-receptor binding is specific. There are several key factors which play an important role in designing an effective magnetic capture system. For instance, the use of small sized MNPs increases the binding kinetics in diffusion-limited processes. Also, a higher surface to volume ratio improves the overall signal by lowering the steric hindrance during ligand-receptor binding due to a larger particle surface curvature. The magnetization of the particle on the other hand, decreases with the particle size making its capture more difficult. It can take up to 20 min to capture 100 nm super paramagnetic MNPs from 1 ml of buffer using a neodymium magnet. In addition to the particle size and magnetic moment, the capture time further depends on the liquid volume and viscous properties. For large volume immunomagnetic capture, the liquid is flowed through a capture zone where an external magnetic field is applied via permanent or electromagnets, micro-patterned magnets in flow channels or self-assembled magnetic fields [23-28]. These magnetic sorting systems require pumps, valves and tubings which increases the complexity and cost of operation. Also, the processed sample cannot typically be taken out of the microfluidic chip for further examination.

To address these issues, we have developed a fully-automated, portable immunomagnetic cell capture device called iMC^2, which by virtue of its unique design can enrich specific targets up to 100× by processing 5 ml samples in just 15 min without using any pumps and tubing accessories. In conjunction with culture enrichment, the device allows reduction in the overall detection time for specific bacterial pathogen identification 48 h to about 7 h, when the lateral flow assay (LFA) is used as a final detection system (**Figure 1**). The device performance is validated using *S. typhi* spiked in PBS buffer and whole blood. We discuss below the detailed analysis of the equipment design and its performance characteristics.

MATERIALS AND METHODOLOGY

Materials used: 100 nm dextran-coated MNPs with free carboxyl groups at the surface (FluidMAG-CMX, Chemicell), polyclonal antibody against *Salmonella spp* (Rabbit antisera, Difco™, BD), LFA strip (Singlepath® *Salmonella,* Merck), tryptone soy broth (TSB); phosphate buffer saline

(PBS); N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC); *N*-Hydroxysuccinimide (NHS); bovine serum albumin (BSA); uranyl acetate from Sigma-Aldrich, Tween 20 (Merck), poly dimethoxysiloxane (PDMS) (Sylgard® 184 silicon elastomer, Dow corning, USA), 300-square mesh carbon-coated copper grids (Electron Microscopy Sciences), neodymium magnets with 300 mT surface field from local hardware shop, 94 primer from 3M and Fevikwik instant adhesive.

Antibody-conjugated MNP preparation: The immunomagnetic nanoparticles (MNP-Ab) were prepared using a standard EDC-NHS protocol. 100 µl of 10^{13} MNPs were washed twice with 10 mM PBS buffer at pH 7.3, followed by incubation with 20 mg/ml each of EDC and NHS for 15 min at room temperature with gentle shaking. The particles were again washed twice with 10 mM PBS buffer pH 7.3 and then incubated with 50 µg of antibody overnight at room temperature with gentle shaking. The suspension was washed twice to remove the unbound antibodies and suspended in PBS buffer. To block any non-specific binding, the particles were incubated with 1% BSA and 0.1% Tween 20 for 1 h at room temperature and stored at 4 °C.

Sample preparation: *S. typhi* was cultured in TSB medium for 12 h at 37° C and based on the optical density (O.D.) at 600 nm the culture was serially diluted from 10^8 to 1 CFU/ml in either 10 mM PBS buffer at pH 7.6 or blood. Whole blood was obtained from a registered blood bank. The concentration of *S.typhi* above 100 CFU/ml in samples was confirmed by multiple plating on tryptone soy agar plates followed by colony counting after 12 h. Samples with *S.typhi* concentrations below 100 CFU/ml were prepared using serial dilution from higher concentrations. A 2.5 ml of spiked buffer sample was added to an equal volume of TSB media and incubated for 8 h at 37° C. Similarly, 1 ml of spiked blood sample was incubated with 9 ml of TSB media for 6 h at 37° C. After media incubation step, 10^{11} MNP-Abs in suspension were added to 5ml of the sample and allowed to incubate for 20 min at room temperature. Binding of MNP-Ab to *S. typhi* was confirmed by transmission electron microscopy (Tecnai TF20, FEI Company) after negative staining with uranyl acetate [29]. The incubated sample was then processed using the iMC^2 device. A fraction of the sample was taken before and after the immunomagnetic capture for plating on tryptone soy agar plates to estimate the number of *S. typhi*.

Design features of the iMC^2 system: The iMC^2 system comprised of a single-use capture chip and an automated portable device. The capture chip, fabricated using CNC machining from a master mould having its negative impression in aluminium, consisted of three layers (**Figure 1A & B**): a top PDMS layer, a middle Parafilm®M sheet layer and a bottom polyacrylic sheet layer. The PDMS solution was prepared by mixing the monomer and the curing agent in 9:1 volumetric ratio as instructed by the manufacturer. The mixture was centrifuged at 2000 RCF to remove any trapped air and then poured into the mould and baked at 60°C for 30 min. The solidified design was removed safely out of the mould and glued first to a parafilm® layer and then to a polyacrylic sheet to make an enclosed capture chip. The PDMS layer had two chambers separated by a manual valve. The valve could be operated in either open or close position to control the flow of liquid between the two chambers. The large 5 ml chamber, referred to as the sample chamber, was used for loading the cultured samples pre-incubated with the MNP-Abs. The smaller chamber of volume 50 µl called the recovery chamber was where the enriched target *S. typhi* cells were collected after the immunomagnetic enrichment. The middle layer of the chip was a 100 µm thick sheet of parafilm®M added to prevent any nonspecific adsorption of the target biomolecules to the surface. The bottom layer provided the mechanical integrity of the chip and was fabricated from a 0.2 mm thick polyacrylic sheet. All the three layers were bonded together with an instant adhesive glue. A primer solution was applied to parafilm® layer before using instant adhesive to make leak proof bonding.

The automated iMC^2 device (**Figure 1C and 2D**) had a magnetic cassette to apply a high gradient magnetic field on the sample, a linear positioner to orient the magnetic cassette and a microprocessor controller for automating the entire process. The magnetic cassette was made by embedding 36 identical cuboidal permanent neodymium magnets of cross-section 10 mm × 10 mm × 10 mm in a 6 × 6 matrix format in a board. The neighbouring magnets were arranged with opposite polarity to each other (i.e., having alternating north/south poles). At one corner of the square magnetic arrangement, 2 circular permanent magnets of 5 mm diameter each were aligned along the square diagonal. The overall size of the matrix arrangement was such that it covered the entire sample chamber and the additional two circular magnets required to sweep the magnetic particles into the recovery chamber. The capture device was fitted with a

microprocessor controller and a linear positioner to control the mechanical movement of the magnetic cassette in the forward and backward directions. The microprocessor could be adjusted to control the translation velocity as required and also allowed real-time display of the steps during the enrichment process. The device also had control buttons to start, stop, and reset the device at any time.

LFA experiments: The 50 µl of immuno enriched sample from the recovery chamber was applied to the sample zone of the LFA strip. The results were visualized after 10 min in the result window that had a test line marked "T" and a control line marked "C" (Figure 1). The occurrence of bands in both "T" and "C" confirmed the presence of *Salmonella* species in the test sample. The occurrence of a band in the "C" line alone indicated a negative result or the absence of *Salmonella* species in the sample. The absence of bands in both "C" and "T" indicated a procedural error and required re-testing. All experiments were performed in triplicate.

RESULTS AND DISCUSSION

i) Rationale for using *S. typhi* as model cells in this study. Disease diagnosis at an early stage can become quite a challenge if the concentration of the pathogen in the biological sample is low. For example, in the case of typhoid, more than 80% of the patients carry the causative agent *Salmonella enterica* serovar Typhi (*S. typhi)* in their blood ≤10 CFU/ml concentration during the early stages of infection [30, 31]. Due to this low number of target cells, culture enrichment of *S. typhi* cells are done for a minimum of 24 to 48 h to reach the detection limit of confirmative tests like lateral flow or agglutination assays. To reduce this turnaround time of culture diagnosis, we investigated the utility of incorporating an immunomagnetic enrichment step.

First, *S. typhi* sample was inoculated into culture media and incubated for 8 hours. After culture enrichment, magnetic nanoparticles labelled with polyclonal antibody (MNP-Ab) against *Salmonella spp.* was added to the sample and incubated for 20 minutes. After incubation, 5 mL of the sample with MNP-Ab mixture was loaded onto the capture chip and placed on the iMC^2 device. Target cells bound with MNPs were captured under high magnetic field gradient and swept from a high volume sample chamber (5 ml) to a smaller volume recovery chamber (50 µl) in capture chip. The enriched sample with higher concentration of target cells was applied on a

lateral flow immunoassay strip. The colour test line in the result window confirms the presence of target microbe in the sample. A schematic diagram outlining the assay protocol is shown in **Figure 2**.

ii) Automated sample processing using iMC^2: Up to 5 ml of the sample mixture, spiked, incubated at 37^{o}C for 6 to 8 h and pre-incubated with MNP-Abs, was loaded into the disposable capture chip fitted directly above the magnetic cassette such that the recovery chamber and the two circular magnets were placed diagonally opposite to each other. The iMC^2 device was then immediately switched on. After waiting for 5 min, the magnetic cassette was moved at 1 cm/min velocity using a stepper motor while keeping the position of the capture chip fixed at all times (**Figure 3**). This linear movement of the magnetic cassette was stopped once the recovery chamber came directly above the last circular magnet. This complete sweep cycle took about 10 min. The valve connecting the sample and the recovery chambers was closed at this stage and the enriched sample (~50 µl in volume) was collected from the recovery chamber for further analysis.

iii) Immunomagnetic cell capture by the sweep method: After sample loading, the immunomagnetic capture was first allowed to happen in the vertical direction by placing the capture chip directly above the magnetic cassette. Within 5 min, all the free and bacteria-bound MNPs were attracted to the bottom of the chip surface in a checker board arrangement (**Figure 3A**). This happened because the magnetic force acting on an MNP (or bacteria bound with MNPs) is proportional to $(B.\nabla)B$, where B is the magnetic flux density and ∇B represents the gradient of the magnetic field. The magnetic field gradients generated by virtue of the opposite polarity magnets were highest between the magnets along the gridlines and thus strongly attracted all the MNPs towards them. The time of vertical capture was empirically optimized and could be increased or decreased depending on the size and magnetic polarizability of the particles, and the physical properties of the bulk liquid (For more details see supplementary section, Figure S1 and Figure S2).

After the vertical capture, the magnetic cassette was moved horizontally by a linear positioner in such a way that the total surface area under the magnetic field was gradually

reduced. This allowed the MNPs to be swept along the highest magnetic field and get more and more concentrated towards the recovery chamber (**Figure 3B**). This translational sweeping movement was continued for approx. 10 min until all the MNPs were collected in the 50 µl volume recovery chamber directly above the last circular magnet (**Figure 3C**).The valve connecting the sample and recovery chambers was then closed to stop the diffusional flow of the MNPs in the reverse direction and enable easy recovery for further processing. Having a bottom surface with minimum non-specific absorption was a crucial factor in the device design to maximize the recovery of the MNPs.

iv) Culture growth and MNP-tagging of *S. typhi* cells: Spiked buffer and blood samples were prepared with different concentrations of *S. typhi* and then incubated with TSB culture media in a specific volumetric ratio. Within 8 h, the buffer samples showed several orders of magnitude increase in CFU/ml as determined by the plating method (**Figure 4A**). When the experiments were, however, repeated with spiked blood samples, the 1:10 blood: media dilution did not yield an efficient bacterial growth (data not shown) possibly due to the antimicrobial properties of blood. So, the blood was further diluted with the media 10× in order to minimize the inhibitory effects of blood. Using this, a growth performance similar to that of PBS buffer spiked data was achieved (**Figure 5A**). With 8 h incubation, *S. typhi* spiked in buffer undergo on average 14 doubling cycles and with 6 h incubation of blood spiked samples ~11 doubling cycles could be achieved (**Figure S3 A&C**). Number of cells increase by a factor of ~ 1.6×10^4 for 8 h incubation and ~ 2×10^3 times for 6 h incubation. Next, the culture-grown samples were incubated with excess antibody-functionalized MNPs. **Figure 4B** illustrates that each *S. typhi* cell was effectively tagged with multiple MNPs. To finally investigate the effectiveness of our approach, this cell-MNP composite mixture was transferred to the capture chip and processed on the iMC^2 device setup.

v) Characterization of the device performance: The capture chip was designed to enrich *S. typhi* concentration per unit volume up to 100× as compared to that in the original sample (5 ml to 50 µl). The concentration of the cells showed a linear dependence between the concentrations of the pre-immuno captured vs. post-immuno captured samples implying that the device performance in terms of capture efficiency remained constant across the entire range of cell

concentrations studied (**Figure 4C**). On average, the iMC^2 device performed with 71.6 ± 11.4 % capture efficiency in spiked buffer samples(**Figure 4D**)and with 68.5 ± 9.3 % in spiked blood (**Figure 5B**). Immuno-capture performance was highly reproducible and statistical distribution of variation from sample to sample is shown as histogram in **Figure S3 B&D**.

vi) LFA results and the lower limit of detection: The detection sensitivity limit of the commercially available LFA strips is known to be 10^7 CFU/ml (**Figure 6A**). Using media and magnetic enrichment steps, number of cells available for detection is improved by factor of ~ 1.1 x 10^6 times with 8 h incubation and ~1.4 x 10^5 times with 6 h incubation. With two stage enrichment process, we show improvement in detection of spiked *S. typhi* samples in buffer using LFA by six orders of magnitude from 10^6 to 4 CFU/ml (**Figure 6B**). In case of spiked blood samples incubated for 6 h and enriched using iMC^2, a positive test result was seen for *S.typhi* count as low as 8 CFU/ml. Intensity of band in test line was faint at lower limits and was completely absent for concentrations below 2 CFU/ml in spiked blood sample (**Figure 6C**). However, data points with initial cell concentration greater than 10 CFU/ml was reliably detected in LFA (Circle points, **Figure 5A**), which lies in the clinically relevant range for typhoid diagnosis. Negative controls performed under similar conditions with up to 10^8 CFU/ml of *E.coli* showed negative results demonstrating the specificity of the immunomagnetic capture method.

CONCLUSIONS

We demonstrate a portable and automated large volume immunomagnetic capture device that can enrich a target / biomarker from blood sample rapidly and with above 65% efficiency. The design of the sample chamber volume (base area × height) in the capture chip is flexible and can be easily modified to increase the total sample processing volume. Our preliminary results show that the total volume of sample used for capture can be scaled even up to 30 ml without significant change in capture time or loss in efficiency (data not shown). Using this large volume immuno capture system combined with culture enrichment, we were able to reduce the time of diagnosis of a specific bacterial pathogen from 48 h (conventional blood culture) to just less than 7 h. The entire approach can be a valuable tool for identification of other pathogens by choosing an appropriate antibody, e.g., *Mycobacterium tuberculosis* where culture diagnosis can take

multiple weeks. In addition, this method could be used to enrich target biomarkers from other biosamples like urine, feces, serum *etc.*, particularly where centrifuge or column based extraction protocols cannot be employed. Finally, the major issue pertaining to drug-resistance development can be addressed by our approach as the patient may be given targeted therapy on the very first day as soon as clinical symptoms start to develop.

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Conflict of interest: Authors have filed an Indian patent as well as a PCT application for the described technology.

Figures

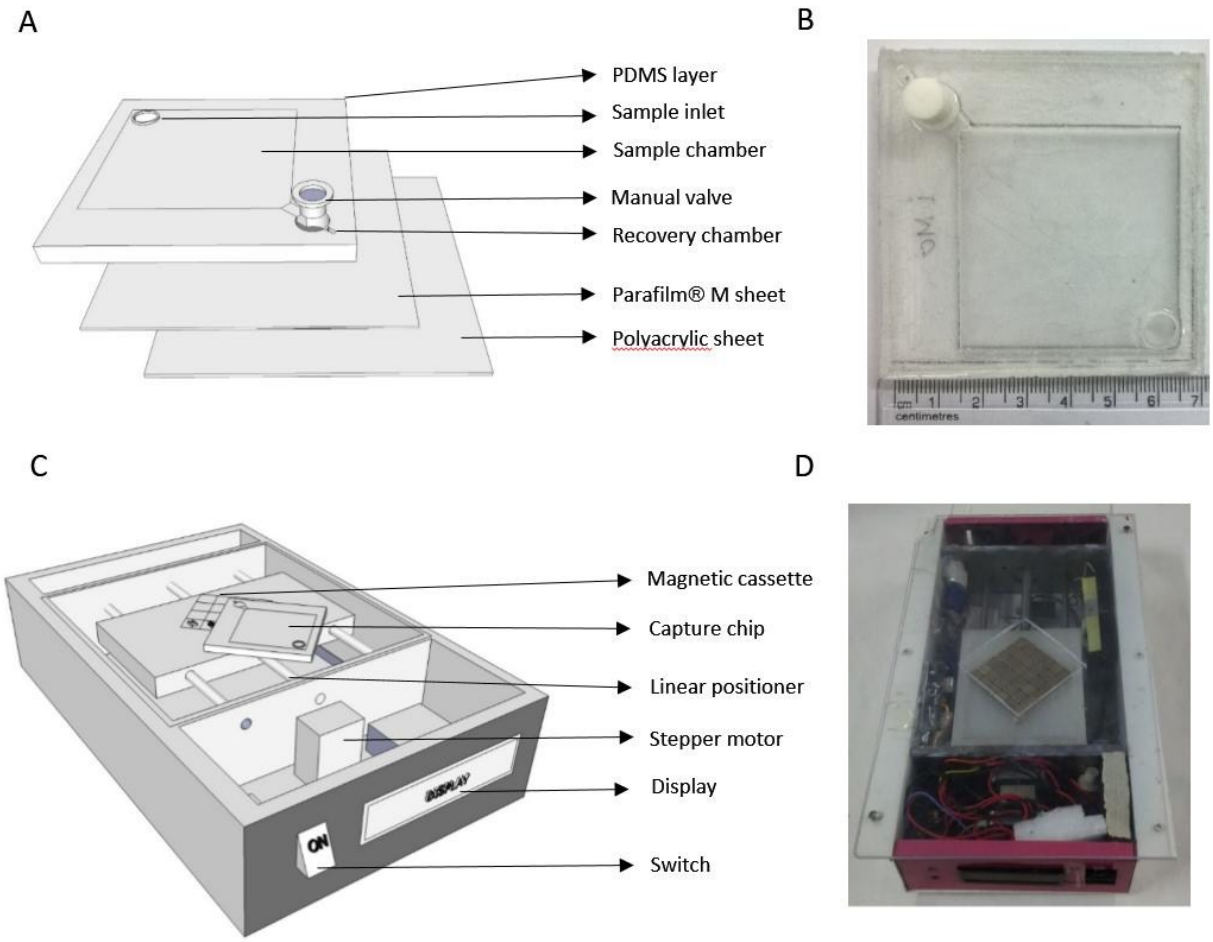

Figure 1: (A) Schematic representation of the capture chip designed to hold and process the MNP-Ab labeled samples. Capture chip is made of 3 layers: (i) *Top:* mold-crafted PDMS layer with

sample port, manual valve, sample chamber and recovery chamber, (ii) *Middle:* parafilm® sheet to avoid non-specific binding of samples, and (iii) *Bottom:* polyacrylic sheet to provide mechanical strength. All three layers were glued together using adhesives. **(B)** Actual view of the capture chip. **(C)** Schematic of the automated device with a magnetic cassette mounted on a linear-positioner connected to a microprocessor-controlled stepper motor. **(D)** Actual image of the iMC^2 device (150 mm ×300 mm: length × width).

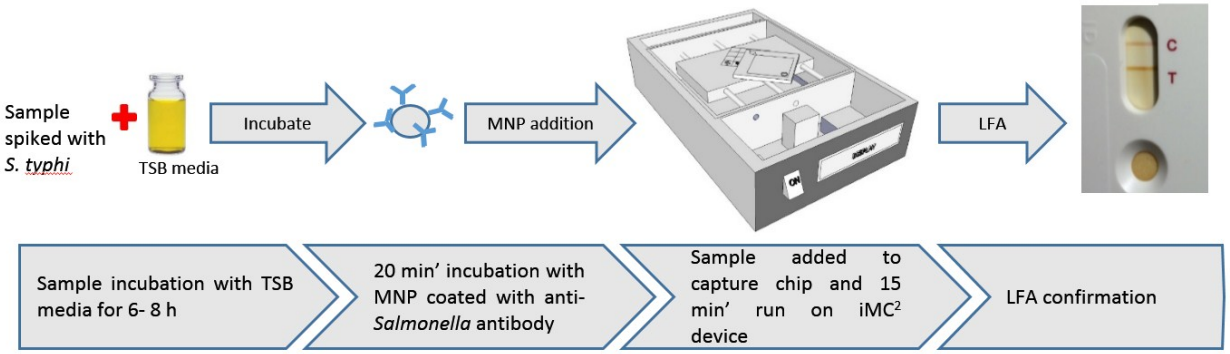

Figure 2: Overall schematic of the immunomagnetic capture enrichment of *Salmonella* cells using the iMC^2 system and result confirmation using an LFA.

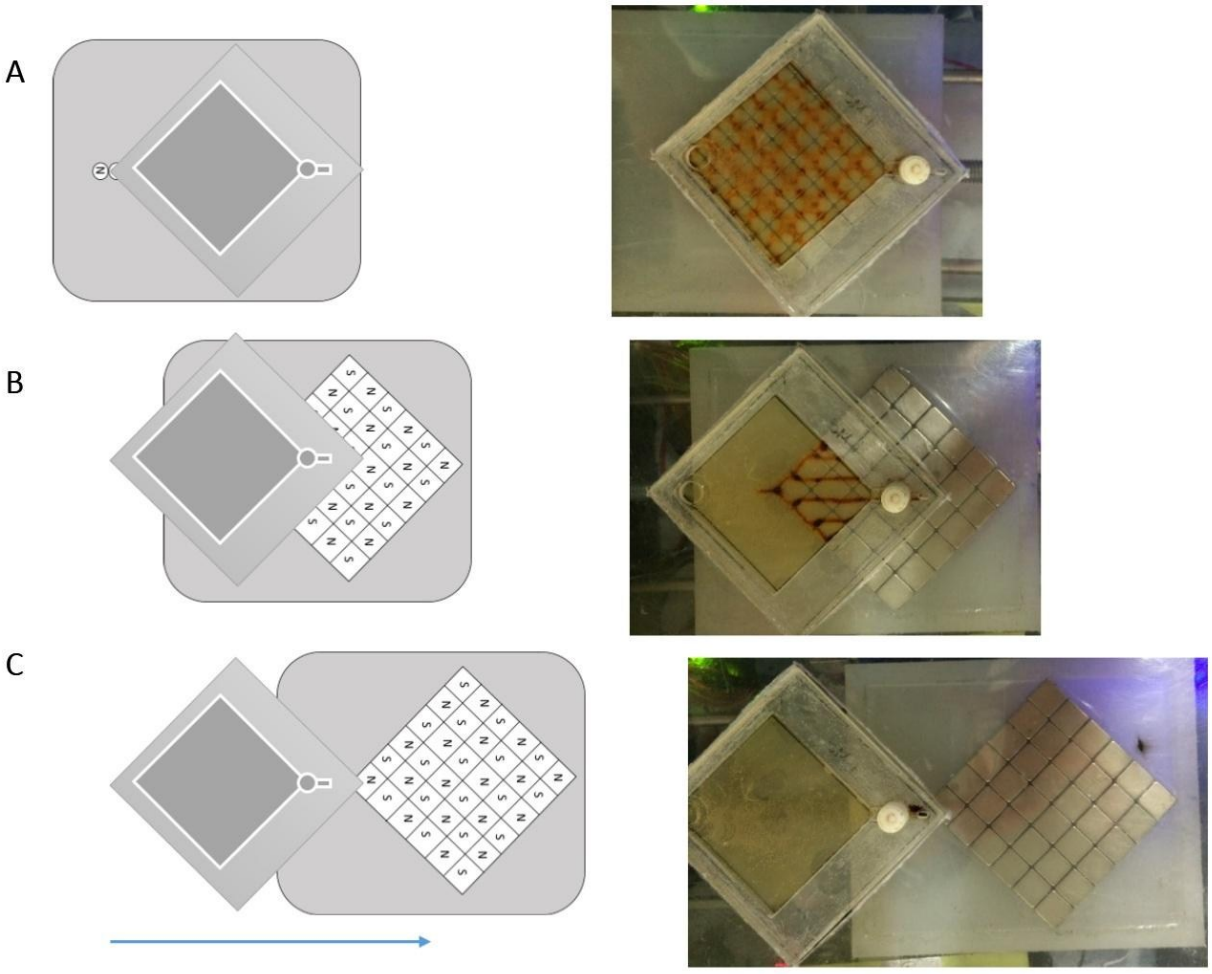

Figure 3: Time-lapsed images of immunomagnetic enrichment process by displacement of the magnetic cassette. **(A)** After 5 min: Cells collected at the highest magnetic field gradients near the bottom surface. **(B)** After 10min: Sweeping action on the MNP-Ab cell complexes as the magnetic field is moved in small steps using a linear positioner. **(C)** After 15 min: Cells concentrated into the 50 µl volume recovery chamber allowing for 100× enrichment.

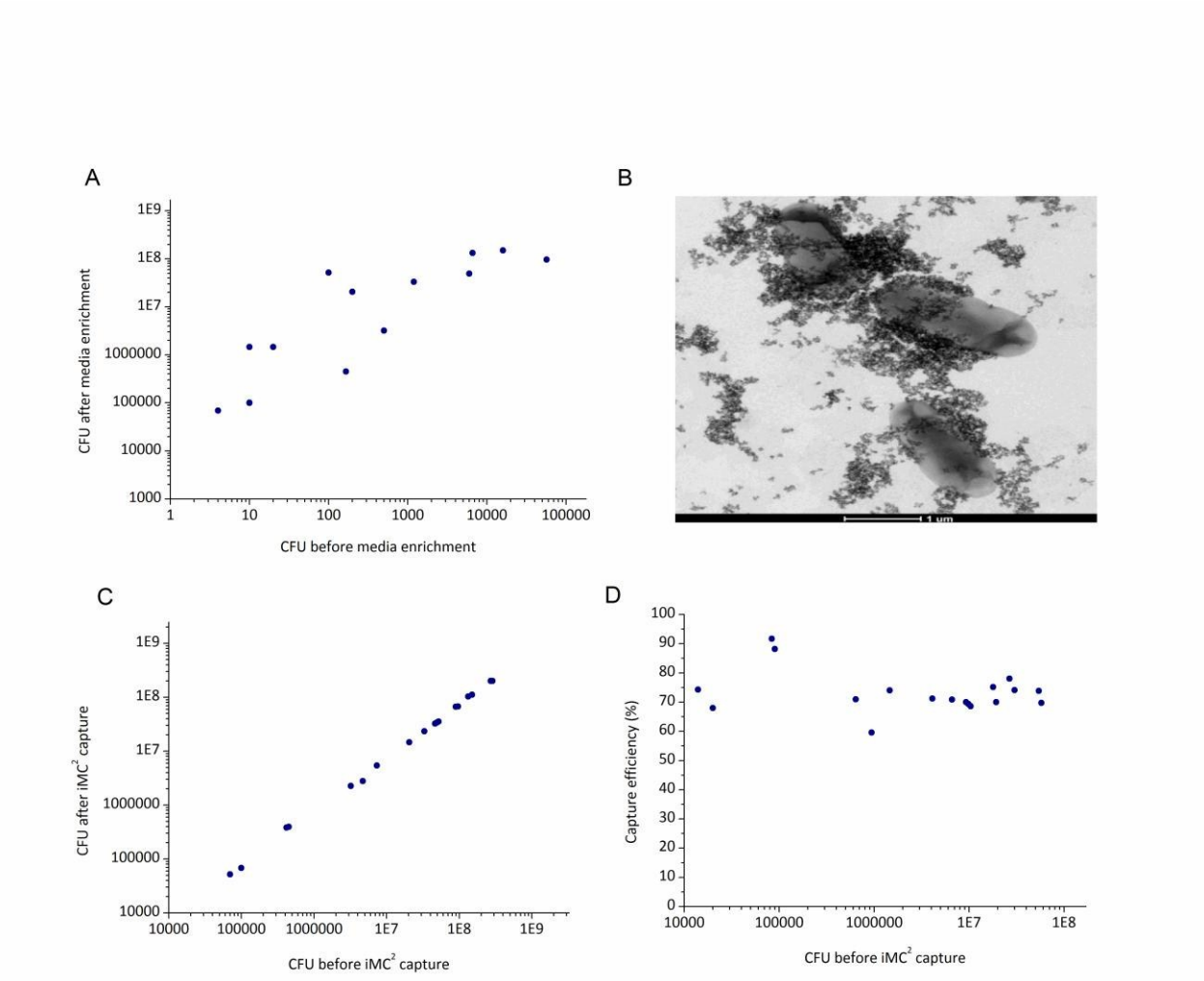

Figure 4: Performance characterization of iMC^2 device with spiked buffer sample: **(A)** Plot of the *S.typhi* counts before and after 8 h incubation in TSB media. **(B)** TEM image of the MNP-bound *S.typhi*. **(C)** Plot of *S.typhi* concentration prior to and after processing with the iMC^2 device. **(D)** Capture efficiency of device calculated by comparing cell count before and after immunomagnetic enrichment.

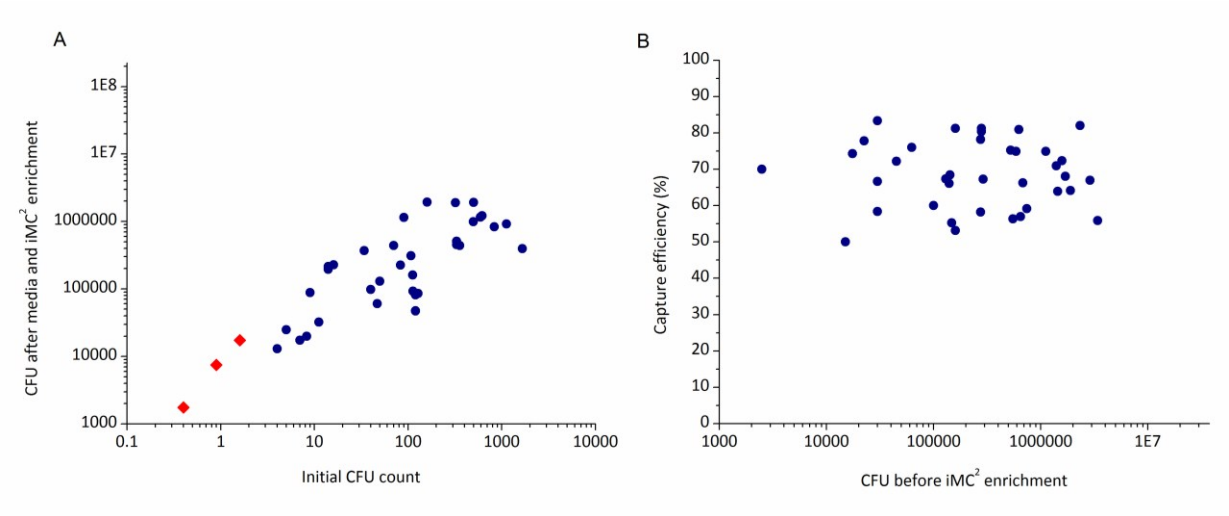

Figure 5: Performance characteristics of iMC^2 device on spiked blood samples: **(A)** Plot of the initial *S. typhi* counts and after 6 h incubation with iMC^2 enrichment. Red diamond points were those that gave negative LFA response. **(B)** iMC^2 capture efficiency calculated by comparing the cell count before and after immunomagnetic enrichment.

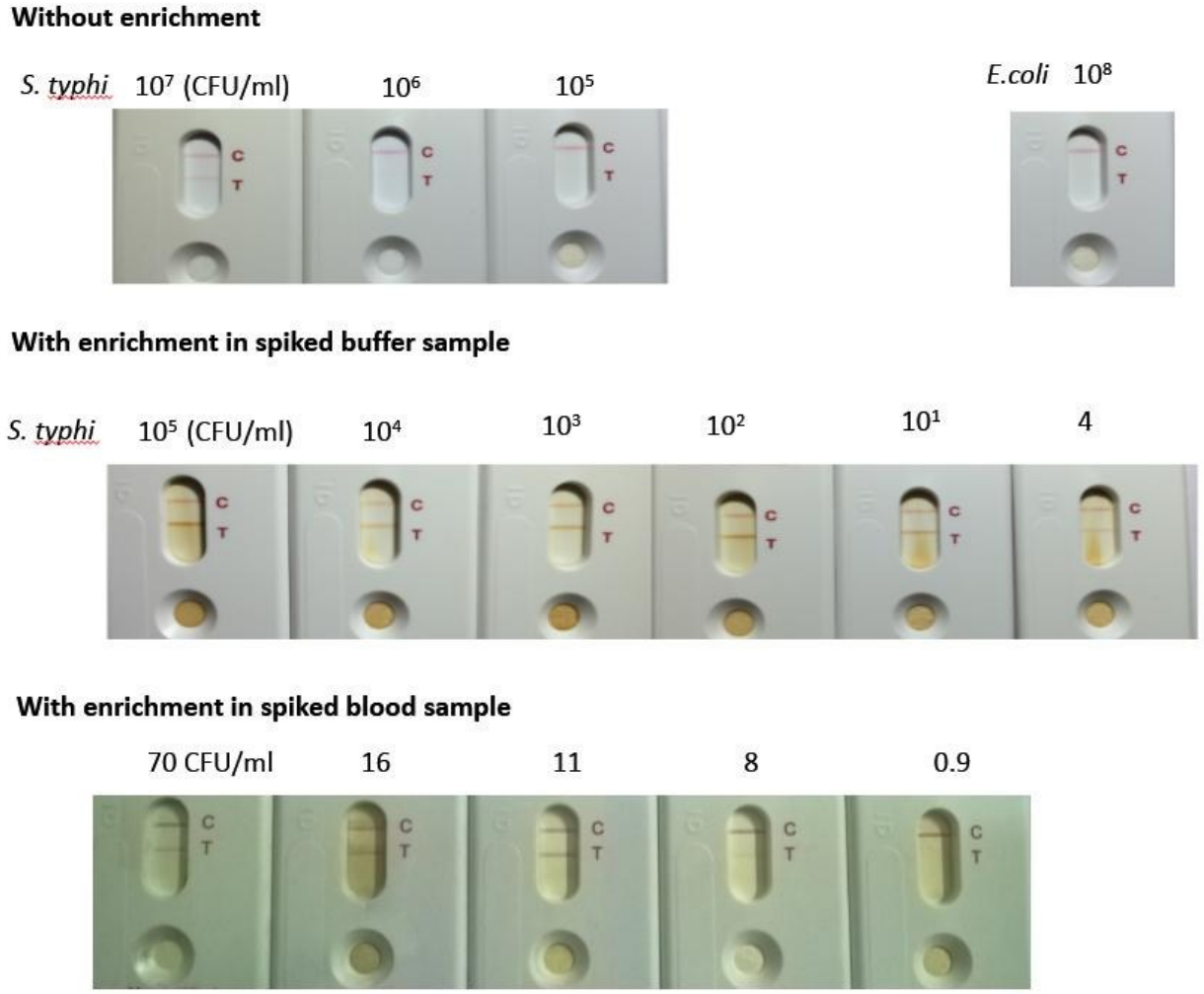

Figure 6: Detection using a commercial LFA. Generation of colored bands in the test window (T: positive, C: control) indicated the presence or absence of *S.typhi* in the samples. **(A)**The LFA used has a lower limit of detection of 10^7 CFU/mL; E.Coli at 10^8 CFU/mL does not give a signal demonstrating the specificity of the LFA. **(B)** Buffer spiked with *S.typhi:* culture media enrichment along with enrichment using iMC^2. Lower than 10 CFU/ml of *S.typhi* was detected in ~9 h. (C) Blood spiked with *S.typhi:* culture media enrichment along with enrichment using iMC^2. Less than 10 CFU/ml of *S.typhi* was detected in ~7 h. These are representative pictures of LFA used for data points in Figure 4 & Figure 5.

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