Microfluidic Chip-Based Detection and Intraspecies Strain Discrimination of *Salmonella* Serovars Derived from Whole Blood of Septic Mice

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*Salmonella* is a zoonotic pathogen that poses a considerable public health and economic burden in the United States and worldwide. Resultant human diseases range from enterocolitis to bacteremia to sepsis and are acutely dependent on the particular serovar of *Salmonella enterica* subsp. *enterica*, which comprises over 99% of human-pathogenic *S. enterica* isolates. Point-of-care methods for detection and strain discrimination of *Salmonella* serovars would thus have considerable benefit to medical, veterinary, and field applications that safeguard public health and reduce industry-associated losses. Here we describe a single, disposable microfluidic chip that supports isothermal amplification and sequence-specific detection and discrimination of *Salmonella* serovars derived from whole blood of septic mice. The integrated microfluidic electrochemical DNA (IMED) chip consists of an amplification chamber that supports loop-mediated isothermal amplification (LAMP), a rapid, single-temperature amplification method as an alternative to PCR that offers advantages in terms of sensitivity, reaction speed, and amplicon yield. The amplification chamber is connected via a microchannel to a detection chamber containing a reagentless, multiplexed (here biplex) sensing array for sequence-specific electrochemical DNA (E-DNA) detection of the LAMP products. Validation of the IMED device was assessed by the detection and discrimination of *S. enterica* subsp. *enterica* serovars Typhimurium and Choleraesuis, the causative agents of enterocolitis and sepsis in humans, respectively. IMED chips conferred rapid (under 2 h) detection and discrimination of these strains at clinically relevant levels (<1,000 CFU/ml) from whole, unprocessed blood collected from septic animals. The IMED-based chip assay shows considerable promise as a rapid, inexpensive, and portable point-of-care diagnostic platform for the detection and strain-specific discrimination of microbial pathogens.

Non-typhoidal *Salmonella* (NTS) is the greatest food-borne-disease burden in the United States, causing the most infections, hospitalizations, and deaths and 1.03 million illnesses annually (1, 2). The economic burden associated with the disease is dramatic, with the medical costs alone reaching $11.4 billion per year and substantial additional costs being incurred by the food industry (recalls, litigation, reduced consumer confidence) and by state, local, and federal public health agencies in response to NTS outbreaks (3). Globally, NTS is estimated to cause 93.8 million cases and 155,000 deaths each year (4), and NTS has emerged as the leading cause of bacteremia in sub-Saharan Africa, where its fatality rate reaches up to 25% (5, 6).

*Salmonella enterica* comprises six subspecies that are subdivided into more than 2,500 serovars (serological variants) on the basis of carbohydrate, lipopolysaccharide (LPS), and flagellar composition, with *S. enterica* subsp. *enterica* encompassing over 99% of human-pathogenic isolates (7–9). *S. enterica* infection can result in any of four distinct syndromes: enterocolitis/diarrhea, bacteremia, enteric (typhoid) fever, and chronic asymptomatic carriage (10, 11). Many serovars infect both humans and animals, whereby disease severity is a function of the serovar, strain virulence, and host susceptibility (10–13). The CDC has recently reported a plethora of *Salmonella* outbreaks in the United States (2011–2012) arising from the consumption of contaminated ground beef, turkey burgers, chicken livers, fish, cantaloupe, mangos, papayas, pine nuts, alfalfa sprouts, dry dog food, and peanut butter or by exposure to any of a wide range of backyard animals (poultry, turtles, frogs, and hedgehogs (14)). The number of illnesses reported during each of these outbreaks may represent only a fraction of the total number of individuals affected, as it is estimated that for every confirmed case, there are as many as 30 that go unreported (1). Thus, an outbreak of 350 reported cases may, in fact, affect more than 10,000 people. Additionally, the false implication of food products can be devastating to a particular industry: tomatoes falsely implicated in the *Salmonella* jalapeno and Serrano pepper outbreak (2008) resulted in losses exceeding $200 million due to a dramatic reduction in tomato consumption (15).

The health and economic burden associated with *Salmonella* appears to be poised to worsen: the prolonged administration of antibiotics has resulted in the emergence of multidrug-resistant NTS strains that have disseminated worldwide. *S. enterica* subsp. *enterica* serovar Typhimurium DT104, for example, which has caused a heightened occurrence of food-borne disease outbreaks over the last 2 decades, is resistant to four of the five most commonly used antibiotics in veterinary medicine (tetracycline, β-lac-
tams, aminoglycosides, and sulfonamides) (16, 17). These multidrug-resistant NTS strains are often more virulent, causing more hospitalizations and bacteremia (18, 19), and their maintenance in nature occurs at very low antibiotic concentrations that are commonly found in the environment, including groundwater (20). Also of concern is the recent isolation of hypervirulent Salmonella from natural microbial populations derived from livestock that are 100 times more virulent than other clinical isolates and are more capable of killing vaccinated animals (21). The NTS disease burden is thus predicted to be exacerbated, with the potential emergence of more potent multidrug-resistant strains that cause an increased health risk due to the lack of available antibiotics to treat infected patients (7, 22–24).

Salmonella control efforts remain challenging for a variety of reasons. Most livestock infections, for example, are subclinical (7, 25); disease outbreaks are sporadic and frequently caused by specific serotypes, although a diversity of serotypes are isolated from livestock production systems (7, 8, 14); and Salmonella survival/proliferation in the environment provides a continuous reservoir for livestock infection and a vehicle for cross-contamination from animal to human food products (26–29). The diversity of Salmonella serovars on farms and feedlots, the human disease manifestation dependence on the particular serovar, and the potential emergence of more potent multidrug-resistant strains highlight the medical need for rapid, reliable, and reproducible methods for strain detection and intraspecies strain discrimination.

A key challenge that prevents the rapid detection of microbial pathogens that cause invasive disease, such as Salmonella, is the often extremely low concentration of organisms in the relevant clinical (e.g., blood) or environmental (e.g., water) samples, which invariably requires amplification of either the bacteria themselves (via culturing) or their nucleic acids (via enzymatic methods) prior to detection (30). Conventional culturing methods are reliable but are laborious, time-consuming, and incompatible with field testing. Nucleic acid-based amplification, such as PCR, is commonly used for Salmonella detection and must be coupled to methods for the detection of amplification products via (i) the generic detection of the formation of double-stranded DNA (31), (ii) the detection of amplification products of specific lengths (32), or (iii) the sequence-specific detection of the amplified product (33, 34). These detection methods, however, are not without significant limitations: the generic detection of double-stranded DNA products can be complicated by spurious amplification, which produces double-stranded product; size-specific detection often requires cumbersome electrophoretic separation techniques that have proven difficult to fully miniaturize (35, 36); and sequence-specific detection has proven complex because traditional PCR amplification produces double-stranded DNA targets ill-suited for detection via hybridization (37). There thus remains a pressing need for convenient methods for coupling nucleic acid amplification with multiplexed, sequence-specific detection.

In response to the arguments presented above, we describe here the development of an integrated microfluidic electrochemical DNA (IMED) chip capable of the detection and intraspecies strain discrimination of closely related Salmonella serovars. Given the potentially wide applicability of such a platform, there are, of course, a myriad of different samples that we could have used to demonstrate its attributes. From among these we selected blood from a mouse model of septicemia because it is highly relevant to clinical diagnostics and because blood has historically proven a very challenging sample matrix (38, 39). Success with this matrix will thus inform the adaptability of the IMED chip to other sample substrates, including those relevant to food safety monitoring.

**MATERIALS AND METHODS**

**Bacterial and animal strains.** Virulent S. Typhimurium reference strain ATCC 14028 (CDC 6516-60) and S. Choleraesuis strain χ3246, obtained from Roy Curtiss III, Center for Infectious Diseases and Vaccinology, Arizona State University, Tempe, AZ (40), were used in all studies. S. Typhimurium is a broad-host-range serovar typically associated with food-borne illness in humans (10, 11). S. Choleraesuis is a highly invasive host-adapted serovar that causes sepsis or extraintestinal focal infections in humans (41) and has recently been shown to generate hypervirulent strain variants during murine passage (21). Both S. Typhimurium and S. Choleraesuis cause a fatal bacteriaemia in mice (21, 40). Bacteria used for these studies were derived from stationary-phase cultures containing Luria-Bertani (LB) medium aerated at 37°C (42). Six- to 10-week-old BALB/c mice were purchased from Charles River Laboratories. All mice were kept under specific-pathogen-free conditions at the Animal Resource Center, University of California, Santa Barbara.

**Ethics statement.** All animal experimentation was conducted following the National Institutes of Health (NIH) guidelines for housing and care of laboratory animals and performed in accordance with institutional regulations after pertinent review and approval by the Institutional Animal Care and Use Committee at the University of California, Santa Barbara.

**Salmonella infection and blood sampling.** Bacterial cells derived from overnight cultures were resuspended in 0.15 M NaCl and administered to BALB/c mice (10⁵ CFU) via intraperitoneal (i.p.) injection (43). Blood (25 to 50 µl) from the tail vein of infected mice was sampled throughout the 5-day infection time course; additionally, blood from cardiac puncture (200 µl) was taken at day 5. Whole blood was collected into heparin-coated tubes (Microvette CB 300 LH; Sarstedt, Germany); 10 µl was used to determine the number of CFU by plating, and the remaining sample was used for IMED chip assays (3 µl per reaction). Whole-blood samples were stored at 4°C for up to 60 days prior to use and were added to the IMED chip assays without further purification/modification.

**LAMP primer design and reaction conditions.** The loop-mediated isothermal amplification (LAMP) primers that we employed are specific for the target region in the recF gene in S. Typhimurium and S. Choleraesuis. The primers were designed with PrimerExplorer v4 software (http://primerexplorer.jp/e/), which selects certain primer sets on the basis of strict parameters, including melting temperature (Tm), resistance to the formation of secondary structure, G+C content, and the distance between primers along the target gene. The primer set that we developed (Table 1) consists of two outer (F3 and B3) and two inner (FIP and BIP) primers.
primers that amplify the recF gene in the vast majority of *Salmonella* serovars but produces products that differ significantly in their single-stranded regions and thus allow strain-specific discrimination. All primers were ordered from Integrated DNA Technologies (Coralville, IA) and used as received.

DNA templates comprising either purified genomic DNA (from a stock of 1 ng/μl diluted in buffer or 200 copies/μl [QiAmp DNA minikit; Qiagen, Chatsworth, CA]) or bacterial cells (10¹⁰ to 10¹¹ CFU/ml diluted in buffer or whole blood) were added to 25 μl (tube reactions) or 35 μl (IMED chip reactions) of a LAMP reaction mixture containing the following: 20 mM Tris buffer, 10 mM KCl, 2 mM MgSO₄, 10 mM (NH₄)₂SO₄, 0.10% Triton X-100, 0.8 M betaine, 1.4 mM (each) the four deoxynucleotide triphosphates, 16 units of Bst DNA polymerase (New England BioLabs, Inc., Beverly, MA), 1% (vol/val) bovine serum albumin, 3.2 μM (each) the inner primers, and 0.4 μM (each) the outer primers. Control reactions performed in micro-Eppendorf tubes were run in a standard thermal cycler at 65°C. IMED chip experiments were run on a heat block at 65°C. LAMP products were analyzed using PAGE (4 to 20% TBE [Tris-borate-EDTA] gel; Invitrogen, Carlsbad, CA) and stained with SYBR Gold (Invitrogen, Carlsbad, CA).

**Electrochemical DNA (E-DNA) probes and synthetic target DNA.** Two oligonucleotide probe sequences complementary to the *Salmonella* reCF gene were ordered from Fidelity Systems, Inc. (Gaithersburg, MD). The 5’ end of these probes was modified with a flexible Letsinger trihexylthiol described previously (44), and the 3’ end of the molecule was modified with a methylene blue (MB) redox reporter (Table 2). To accurately calibrate the sensors for detection of LAMP products, synthetic oligonucleotides equivalent to the smallest expected LAMP products (here termed “barbells,” which describes the secondary structure that they adopt) were also obtained via commercial synthesis (Table 2). An equivalent barbell structure lacking the target sequence was employed as a negative control. These initial calibration tests with synthetic amplicons were performed in 6 M H₂SO₄ and 0.50 V. The IMED chips, which can, in theory, be reused (49), were differentially labeled by incubating each electrode in individual tubes containing either S. Typhimurium or S. Choleraesuis E-DNA probes and passivated with 6-mercaptohexanol in 2× SSC buffer over-night. Prior to use in experiments, the electrodes were allowed to sit in 2× SSC buffer for at least 30 min at room temperature to equilibrate the sensor. Reagent-grade chemicals, including 6-mercapto-1-hexanol, sulfuric acid, and SSC buffer (all from Sigma-Aldrich, St. Louis, MO) were used without further purification.

Design and fabrication of the IMED chips followed protocols described previously (47). The microfabricated gold electrodes on the IMED chip do not require manual polishing steps postfabrication and are prepared using electrochemical cleaning. Since the IMED chips contain both gold pixel surfaces within a single enclosed chamber, differential labeling of the pixels requires a multistep procedure, which includes subsequent immobilization and electrochemical stripping steps (48). First, both pixels on the chip were immobilized with the S. Typhimurium-specific E-DNA probe and passivated with 6-mercaptohexanol in the same manner as the rod electrodes described above. Next, one pixel was selected and a low potential (−1.4 V versus Ag/AgCl) was applied and held for ~90 s to electrochemically strip the gold surface of its monolayer. S. Choleraesuis-specific E-DNA probe was then injected into the chamber to immobilize onto the exposed gold pixel, followed by passivation with 6-mercaptohexanol, also as described above. Square-wave voltammetry (SWV) was performed in 6× SSC buffer between each step to verify that proper absorption/desorption of the monolayer was completed. The rod electrodes were differentially labeled by incubating each electrode in individual tubes containing either S. Typhimurium or S. Choleraesuis E-DNA probes.

**E-DNA sensor and IMED chip measurements.** Electrochemical measurements on rod electrodes and IMED chips were performed on a CHI 660D potentiostat (CH Instruments, Austin, TX). Rod electrodes were measured with a platinum counter electrode and an Ag/AgCl (3 M NaCl) reference electrode. Both rod and IMED chip measurements were taken using SWV at 60 Hz and a 25-mV amplitude over a potential range of −0.05 to −0.50 V. The IMED chips, which can, in theory, be reused (49), are expensive to fabricate and thus were used only a single time to reduce the risk of cross contamination. Labeled gold rod electrodes may

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**TABLE 2 Sequences for *Salmonella* serovar-specific E-DNA target regions, synthetic LAMP target amplicons, and E-DNA probes**

<table>
<thead>
<tr>
<th>DNA and serovar</th>
<th>Sequencea (5’→3’)</th>
<th>Gene position</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-DNA target region</td>
<td></td>
<td>843–864</td>
</tr>
<tr>
<td>S. Typhimurium</td>
<td>CTCGCCCTGACGGCCCGTG</td>
<td>843–864</td>
</tr>
<tr>
<td>S. Choleraesuis</td>
<td>CTCGCCCTGACGGCCCGTG</td>
<td>843–864</td>
</tr>
</tbody>
</table>

**Synthetic LAMP target amplicons**

| S. Typhimurium barbell | GATAAACGAGGCCAACAGCTTCCCGATGTGTTAAGCCAATAGCATAGTCCAGTGTCGATACGGACCTCGCCCGTGAGAACAAACGCTTCGCTGCTGTCGCCCTGCTTATCTTTGGTTAAGCCAATCGACGTCTCACGCTCTTCGC | 843–864 |
| S. Choleraesuis barbell | GATAAACGAGGCCAACAGCTTCCCGATGTGTTAAGCCAATAGCATAGTCCAGTGTCGATACGGACCTCGCCCGTGAGAACAAACGCTTCGCTGCTGTCGCCCTGCTTATCTTTGGTTAAGCCAATCGACGTCTCACGCTCTTCGC | 843–864 |

**E-DNA probe**

| S. Typhimurium | HS-trihexylthiol-CACGGCCGATTACAGACGGAAG-(CH₂)₇-NH-MB | 843–864 |
| S. Choleraesuis | HS-trihexylthiol-CACGGCCGATTACAGACGGAAG-(CH₂)₇-NH-MB | 843–864 |

a Strain differences are in bold, and binding regions are underlined. MB, methylene blue.
be used multiple times. A flush of deionized water for approximately 60 s removes the target DNA from the sensors and regenerates them for future use (49). For all experiments, each data point represents average readings from three independently fabricated sensors or chips. The means and standard deviations were calculated using Microsoft Excel and Kaleida- graph software.

RESULTS

IMED chip for detection and discrimination of Salmonella serovars. S. enterica can cause any of a wide range of human diseases depending on the S. enterica subsp. enterica serovar (10, 11), exemplifying the pressing need for diagnostics capable of rapid detection and serovar discrimination. Toward this goal, we have developed an IMED microfluidic chip capable of performing amplification and sequence-specific, multiplexed detection of Salmonella on a single device. The IMED chip consists of an amplification chamber that supports LAMP, a single-temperature amplification method that produces single-stranded products amenable to hybridization-based detection (for a review, see references 50 to 52). The LAMP amplification chamber is connected via a microchannel that allows dilution and transfer of the complete LAMP products to the electrochemical detection chamber containing a multiplexed (here biplex) sensing array for sequence-specific E-DNA detection of the LAMP products (Fig. 1). Each E-DNA sensor array contains two sequence-specific DNA probes, each of which is specific for a given bacterial strain. Validation of the IMED device was carried out on S. enterica subsp. enterica serovars Typhimurium and Choleraesuis. These two serovars, the causative agents of enterocolitis and sepsis in humans, respectively, are quite closely related (homologous genes of Salmonella serovars have over 97% DNA sequence identity) and thus serve as a particularly challenging test of the specificity of our platform (41, 53–58; reviewed in reference 59).

**LAMP of Salmonella-specific sequences.** A LAMP reaction of Salmonella-specific sequences requires the identification of DNA primers that recognize a region of the Salmonella genome that is sufficiently conserved—such that a single set of LAMP primers amplifies the region in a large subset of pathogenic Salmonella serovars—yet divergent enough that internal sequences within the amplicon can readily discriminate closely related serovars (60–62). To this end, we employed PrimerExplorer v4 software to design a primer set for the amplification of a region in the recF gene, a well-conserved gene encoding the RecF gap repair protein (63, 64). These LAMP primers consist of two outer (F3 and B3) and two inner (FIP and BIP) primers that should amplify the recF gene in Salmonella serovars, producing barbell-shaped DNA molecules possessing target-containing, single-stranded loops varying by 4 bases between S. Typhimurium and S. Choleraesuis (Fig. 2; Table 2). In terms of specificity, we designed these LAMP primers to amplify the recF gene, the sequence of which is >98% identical across the Salmonella serovars Typhimurium, Choleraesuis, Newport, Dublin, and Enteritidis (BLAST nucleotide sequence comparison). Given this, the primer set is expected to amplify a large fraction of all clinically relevant Salmonella serovars. However, the single-stranded regions in the recF gene produced in the resultant amplicons in this study were specifically designed to discriminate S. Typhimurium from S. Choleraesuis.

Before we employed the approach on chip, we explored the LAMP protocol in micro-Eppendorf tubes using S. Typhimurium and S. Choleraesuis genomic DNA. Using the recF-specific primer set on both S. Typhimurium and S. Choleraesuis DNA, LAMP produced the characteristic ladder-like banding pattern (due to products of various sizes) on polyacrylamide gels indicative of positive amplification (Fig. 2B). Of note, although the amplicon products produced differ in sequence from serovar to serovar, they are identical in size, and thus, they cannot be distinguished by electrophoresis or other sized-based detection schemes. We confirmed the specificity of the LAMP reactions via control experiments either lacking template DNA or employing genomic DNA

![Microfluidic Chip-Based Detection of Salmonella](image-url)
prepared from Shigella flexneri (ATCC 12022). As expected, neither of these negative controls resulted in a corresponding ladder pattern (data not shown).

**E-DNA sensor characterization and detection of Salmonella-specific DNA sequences.** For the detection of LAMP products, we have employed serovar-specific linear probe E-DNA sensors (45, 49, 65). These sensors comprise a linear, single-stranded, redox-tagged DNA molecule covalently attached to a gold surface via a self-assembled monolayer (Fig. 1B) (45, 49, 65). Upon hybridization to a cDNA target (e.g., amplified sequences within the recF gene), the redox reporter (methylene blue) located on the distal end of the linear probe is held in a position fixed away from the electrode surface, reducing the observed redox current (Fig. 1C).

E-DNA sensors demonstrate a high level of specificity and selectivity, with mismatch discrimination down to 3 base pairs and detection capabilities in a wide array of clinical samples (e.g., blood, serum, soil, and foodstuffs [49, 66, 67]). In this study, the single-stranded regions produced in the LAMP amplicons, which differ between the serovars. (B) A polyacrylamide gel loaded with triplicate LAMP reactions of S. Typhimurium (lanes 1 to 3) and S. Choleraesuis (lanes 4 to 6). The LAMP products derived from different serovars differ in sequence but are indistinguishable in terms of product lengths. (C) The lowest-molecular-size LAMP products that we detect are ~150-base, barbell-like structures with a single-stranded loop region (in yellow) containing a 22-base, single-stranded sequence complementary to the E-DNA probes (Table 2).

**FIG 2** *Salmonella* recF-specific primers produce LAMP target amplicons that contain an E-DNA target region. (A) A LAMP primer set was designed that amplified a region of the recF gene in *Salmonella* that contains a 22-base, single-stranded E-DNA target region (yellow), shown here for S. Typhimurium. *, bases that differ between the serovars. (B) A polyacrylamide gel loaded with triplicate LAMP reactions of S. Typhimurium (lanes 1 to 3) and S. Choleraesuis (lanes 4 to 6). The LAMP products derived from different serovars differ in sequence but are indistinguishable in terms of product lengths. (C) The lowest-molecular-size LAMP products that we detect are ~150-base, barbell-like structures with a single-stranded loop region (in yellow) containing a 22-base, single-stranded sequence complementary to the E-DNA probes (Table 2).
Microfluidic Chip-Based Detection of Salmonella

FIG 3 Salmonella E-DNA sensors exhibit rapid and sensitive equilibration kinetics and can discriminate Salmonella serovars. IMED chips were fabricated to contain E-DNA sensors for the detection of both S. Typhimurium and S. Choleraesuis. (A) The analytical sensitivity of each E-DNA sensor was examined by challenging with synthetic LAMP target amplicons. (B) The equilibration kinetics of the sensors was characterized by injecting saturating (100 nM) concentrations of their cognate synthetic target amplicon. (C) The cross-reactivity of each sensor was examined by challenging it with saturating concentrations (100 nM) of its own target, the target of the other sensor, and a non-specific DNA that neither sensor targets (Table 2). All electrochemical measurements in this figure and Fig. 4 and 5 were made using a CHI 660D potentiostat (CH Instruments, Austin, TX) (see Materials and Methods). The data points and error bars represent the means and standard deviations of measurements from three independently fabricated sensors/chips. Measurements of numbers of CFU/ml were determined by direct colony counting.

control value (Fig. 3C). This said, some cross-reactivity is seen when the S. Typhimurium sensor is challenged with the S. Choleraesuis target due to the nonzero affinity of this target-probe pair; the two serovars that we have used in our study are very closely related, and thus, there is significant complementarity even between the incorrect probe-target pair. No signal was observed when the E-DNA sensors were tested with synthetic barbell targets that possess a single-stranded loop region containing a sequence nonspecific (Table 2) to either sensor (Fig. 3C).

IMED chip characterization of Salmonella serovars in rat blood. Before initiating the murine model of Salmonella for testing, we performed preliminary validation of the IMED device with LAMP on commercially available rat blood samples with the addition of ~1,000 CFU/ml of either or both S. Typhimurium and S. Choleraesuis. Spiked blood samples (3 μl each) were added to the LAMP reaction components (35 μl total) and injected into the LAMP chamber on the chip. The chip was set on a heat block for 90 min, and the resulting products were pushed via buffer injection into the electrochemical detection chamber, where the E-DNA sensors reside for electrochemical measurement. IMED chips loaded with blood spiked with both S. Typhimurium and S. Choleraesuis generated a signal response for both E-DNA sensors, with responses of 41% ± 1% on the S. Typhimurium E-DNA sensor and 32% ± 3% on the S. Choleraesuis E-DNA sensor (Fig. 4). To discern the level of cross-reactivity between these two serovars, the IMED chips were loaded with blood spiked with either S. Typhimurium or S. Choleraesuis. The IMED chips responded only to the particular serovar loaded into the IMED chip (Fig. 4). No signal was observed when E-DNA sensors were tested in LAMP buffer with uninfected blood. Note that negative signals can arise when large amounts of nonspecific DNA are applied. Nontarget DNA can interact with the E-DNA sensors in a nonspecific manner near the electrode surface, as is the case when the S. Choleraesuis LAMP target is applied to an S. Typhimurium E-DNA sensor. These data indicate that the IMED chip-based assay is capable of detecting both Salmonella serovars at once with minimal cross-reactivity between S. Typhimurium and S. Cholerae-
suis, such that discrimination between the serovars is evidenced by distinct E-DNA sensor responses.

Clinically sensitive detection and identification of *Salmonella* serovars in whole, unprocessed blood from septic mice. To demonstrate the clinical utility of the IMED chip, we tested the performance of the assay on the detection and discrimination of closely related *Salmonella* serovars in a murine model of typhoid fever, causing a fatal bacteremia resulting in bacteria in lymphoid tissues, blood, and visceral organs. BALB/c mice were infected via the intraperitoneal route of infection with a lethal dose (1,000 CFU, which is 100 times the 50% lethal dose) of either *Salmonella* reference strain ATCC 14028 or *S. Choleraesuis* strain χ3246 (21, 40). Blood (25 to 50 µl) was collected from the tail vein of mice at day 5 postinfection (late stage), 3 µl of which was added to the LAMP reaction components (total volume, 35 µl), and the mixture was injected into the amplification chamber of the IMED chip. An additional 10 µl was reserved to determine the number of CFU by direct colony counting upon plating (detecion limit, ~100 CFU/ml). Using these samples, we found that the IMED chip assay can detect and discriminate between *S. Typhimurium* and *S. Choleraesuis* serovars derived from whole, unprocessed blood, where the bacterial load ranged from $8 \times 10^2$ to $6.9 \times 10^4$ CFU/ml (Fig. 5). To assess the specificity of the IMED chip, we analyzed blood from uninfected mice and mice infected with *Yersinia pseudotuberculosis*, which also causes fatal bacteremia. The IMED chip assay detected little or no signal from blood samples from uninfected mice or from septic animals with *Yersinia* bacteremia at bacterial loads as high as $1.1 \times 10^4$ CFU/ml. These data indicate that the IMED chip assay is specific for *Salmonella* sequences in blood samples from septic mice.

The sensitivity of the IMED device was compared to that of direct colony counting by using both detection methods on blood collected from the tail vein of i.p. infected mice throughout the 5-day infection time course. *Salmonella* bacteremia was not detected by either method until day 5 postinfection (late stage), when the bacterial load in the blood ranged from $10^{-2}$ to $10^5$ CFU/ml (Fig. 6A). These data indicate that both methods are equally sensitive for detecting *Salmonella* bacteremia, where the bacterial load in the blood is extremely low until late stages of infection (limit of detection by plating, ~100 CFU/ml). In contrast, colonization of the liver and spleen was more rapid and progressed to very high levels throughout the 5-day time course postinfection (Fig. 6B). Thus, although IMED can rapidly (within 2 h) detect *Salmonella* at clinically relevant blood concentrations of $<1,000$ CFU/ml, these findings exemplify the challenge of detecting invasive pathogens that are often present in the blood at extremely low concentrations until end-stage infection, even though mucosal and systemic tissues may be highly colonized at much earlier stages of infection. Moreover, the microbial infection kinetics of blood and host tissue sites may vary widely between and within microbial species (21), posing an additional challenge to the design and implementation of blood-based pathogen detection systems.
DISCUSSION
The FDA and USDA have made the development of improved methods for *Salmonella* monitoring from farm to fork a major priority to limit the size, frequency, and severity of food-borne outbreaks as well as to reduce the false implication of other food products to avoid catastrophic economic losses (15, 68–70). Improved monitoring of salmonellae will prevent contamination of food and water supplies at the outset and, in turn, significantly reduce pathogen exposure, transmission, animal disease, and the direct contamination of livestock-derived food products as well as the indirect contamination of fruit and vegetable food products via contaminated water. In response, we have established the utility of a microfluidic IMED chip to distinguish two closely related *Salmonella* serovars using blood samples derived from a murine model of septicemia. The extensive DNA similarity between *Salmonella* serovars (homologous genes having greater than 97% DNA sequence identity; reviewed in reference 59) serves as a demanding test of the specificity of our platform, and blood has proven to be a very challenging sample matrix. The IMED device facilitated rapid (<2 h) detection and intraspecies strain discrimination of *S. Typhimurium* and *S. Choleraesuis* directly from undiluted, unmodified whole-blood samples derived from infected animals at clinically relevant concentrations (<1,000 CFU/ml), pushing preparation requirements to an absolute minimum. The IMED platform is readily adaptable to diverse sample substrates in a number of potential commercial applications, including those relevant to clinical diagnostics (blood, urine, stool), food safety (stool, food matrices), and environmental/farm management (feed pack, soil, surface water).

The IMED chip is an affordable assay—we estimate that current fabrication costs are about $15, with the potential for $1-per-chip costs with larger batch preparations—and can be configured for the simultaneous detection of multiple pathogens by changing the LAMP amplification primer set and the E-DNA probe sequences. This is evidenced by the clinical utility, specificity, and sensitivity of the LAMP reaction that has been established for the detection of pathogens, including *Mycobacterium tuberculosis* from sputum (71), *Plasmodium falciparum* (72) and *Trypanosoma brucei* (73) from blood, and dengue virus from serum (60). There have also been recent developments in the private sector, as Eiken Chemical Company is releasing two new clinical LAMP assays in the near future (74). The first is the Loop amp tuberculosis complex detection reagent kit for the detection of *M. tuberculosis* from untreated sputum samples. The second is a field-deployable test for human African trypanosomiasis, which is currently entering on-site field trials in Uganda and Congo with expected release later in 2013. Collectively, these LAMP studies provide a solid foundation for the development of the relatively nascent field of nucleic acid-based, point-of-care, or even field-portable pathogen detection.

The IMED diagnostic platform may have utility in hospitals as well as makeshift medical facilities for strain discrimination of a variety of pathogens. *Streptococcus pneumoniae*, for example, is a major cause of pneumonia, sepsis, and meningitis in the developed and developing world (75), and patient disease outcome is significantly improved if treatment occurs before the onset of the thrombohemorrhagic pathology of sepsis termed disseminated intravascular coagulation (DIC)—or “death is coming,” in physicians’ vernacular. There are more than 90 pneumococcal capsular serotypes, each exhibiting a different prevalence in human populations and clinical disease outcome (i.e., pneumonia, septicemia, meningitis, infective complications of chronic obstructive pulmonary disease [76–78]). Improved methods for early detection and discrimination of *S. pneumoniae* capsular serotypes would thus be a major advance for clinicians and could be potentially achievable using the IMED device based on nucleic acid sequence differences between clinically relevant strains.

A key challenge that prevents the rapid detection of pathogens is the often extremely low concentration of pathogens in the blood. Clinically relevant HIV loads, for example, can be lower than 50 copies/ml in blood plasma (79, 80), and bacterial loads in the blood of septicemic patients are rarely above 1,000 CFU/ml (81). This is supported by our findings in a murine model of typhoid fever, in which blood CFU levels are below the detection limit of IMED or direct colony count methods until end-stage infection (limit of detection by plating, ~100 CFU/ml). Patients may harbor a low pathogen load in the blood, and the capacity for early detection and monitoring the pathogen load in real time would significantly improve the utility of IMED. One limitation of the current IMED architecture is its rather small chamber size; LAMP amplification fails in whole blood at dilutions of more than ~10%, and thus, the 35-μl volume of the LAMP chamber on our first-generation IMED chip precludes the detection of bacterial loads of less than ~1 CFU/3.5 μl or ~350 CFU/ml. Given that this is purely a volumetric constraint, a severalfold improvement in the detection limit of the IMED chip should be easily realizable. Further, recent advances in IMED technology, termed microfluidic electrochemical quantitative loop-mediated isothermal amplification (MEQ-LAMP), have resulted in an integrated microfluidic platform for the rapid, sensitive, and quantitative detection of pathogenic DNA in real time, which should prove useful in the clinical setting (82).

Point-of-care pathogen detection methods would have significant utility in medical, veterinary, and field applications in cases wherein rapid, on-site diagnosis is not feasible (cases of acute illness, nonambulatory patients/animals, contaminated environment), expensive, or time-consuming. Such methods would be particularly useful if compatible with (i) a wide range of clinical and environmental samples (e.g., blood, feces, urine, sputum, masure, feedlot effluent), (ii) varied surfaces (for testing the effectiveness of methods of disinfection of hospital equipment and food processors), and (iii) the discrimination of clinically relevant strain variants associated with an increased incidence and severity of disease. Implementation of the IMED device in risk-management strategies may promote human and animal health, reduce contamination of livestock-derived food products, and enhance food safety.

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