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A culture-free biphasic approach for sensitive and rapid detection of pathogens in dried whole-blood matrix

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Blood stream infections (BSIs) cause high mortality, and their rapid detection remains a significant diagnostic challenge. Timely and informed administration of antibiotics can significantly improve patient outcomes. However, blood culture, which takes up to 5 d for a negative result, followed by PCR remains the gold standard in diagnosing BSI. Here, we introduce a new approach to blood-based diagnostics where large blood volumes can be rapidly dried, resulting in inactivation of the inhibitory components in blood. Further thermal treatments then generate a physical microscale and nanoscale fluidic network inside the dried matrix to allow access to target nucleic acid. The amplification enzymes and primers initiate the reaction within the dried blood matrix through these networks, precluding any need for conventional nucleic acid purification. High heme background is confined to the solid phase, while amplicons are enriched in the clear supernatant (liquid phase), giving fluorescence change comparable to purified DNA reactions. We demonstrate single-molecule sensitivity using a loop-mediated isothermal amplification reaction in our platform and detect a broad spectrum of pathogens, including gram-positive methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* bacteria, gram-negative *Escherichia coli* bacteria, and *Candida albicans* (fungus) from whole blood with a limit of detection (LOD) of 1.2 colony-forming units (CFU)/mL from 0.8 to 1 mL of starting blood volume. We validated our assay using 63 clinical samples (100% sensitivity and specificity) and significantly reduced sample-to-result time from over 20 h to <2.5 h. The reduction in instrumentation complexity and costs compared to blood culture and alternate molecular diagnostic platforms can have broad applications in healthcare systems in developed world and resource-limited settings.

blood stream infection (BSI) | sepsis diagnosis | biphasic | porous dried blood matrix | isothermal amplification

Significance

Antibiotic therapy within 3 h of initial symptom-based recognition can significantly reduce mortality in blood stream infections and bacteremia. However, blood cultures, which remain the gold standard for pathogen detection, can take up to 5 d for a confirmed negative result. Here, we report a culture-free "biphasic" approach to performing amplification reactions directly from whole blood. We dry the blood and create a physical nano scale fluidic network inside the dried blood matrix to allow for DNA amplification. We show single-molecule sensitivity for 3 bacteria and 1 fungal species from ~1 mL of blood in <2.5 h. We validated the assay with clinical samples and found complete agreement with the results of the clinical laboratory that used blood culture and PCR.

Fast and accurate identification of infection-causing microorganisms in blood remains a significant diagnostic challenge (1). Blood stream infections (BSIs) are often associated with severe diseases and result in high morbidity and mortality, especially in critically ill patients (2). Sepsis, a life-threatening organ dysfunction caused by a dysregulated host response to infection, is the most important diagnostic and therapeutic challenge from BSIs (3). Sepsis is currently the most expensive condition treated in US hospitals, with its nonspecific diagnoses alone accounting for US $23.7 billion every year (4, 5). Despite that, there was an alarming 31% increase in sepsis-related deaths between 1999 and 2014 (6). Moreover, neonates comprise an additional group vulnerable to BSIs due to their deficient adaptive immune responses. Twenty-five percent of all neonates admitted to the neonatal intensive care unit (ICU) are diagnosed with sepsis, and 18 to 35% end up dying from pathogen infection (7–9).

Sepsis generally results from a primary bacterial infection or less frequently from fungal and/or viral infection (1). It has been shown that timely administration of appropriate antibiotics significantly improves patient outcomes (10, 11). However, the current clinical gold standard for diagnosing sepsis/BSIs remains blood culture followed by nucleic acid amplification and detection using PCR. The blood culture step is too slow and cumbersome to allow for initial management of patients and thus contributes to high mortality (12–14). Moreover, in the absence of timely results from robust diagnostic tests, the patients are administered highly potent broad-spectrum antibiotics without any patient stratification, increasing antimicrobial resistance and emergence of drug-resistant and atypical pathogens (15, 16).

Blood culture for diagnosing BSIs requires the culture of viable pathogens for up to 5 d (Fig. 1*A*) (1, 17). If the culture is positive, then morphological and molecular testing is performed to identify the pathogen (1) (Fig. 1*B*). However, it has been shown that correct initial choice of antibiotic therapy, specifically within 1 to 3 h from the initial symptom-based sepsis recognition, has a higher contribution to reducing mortality than

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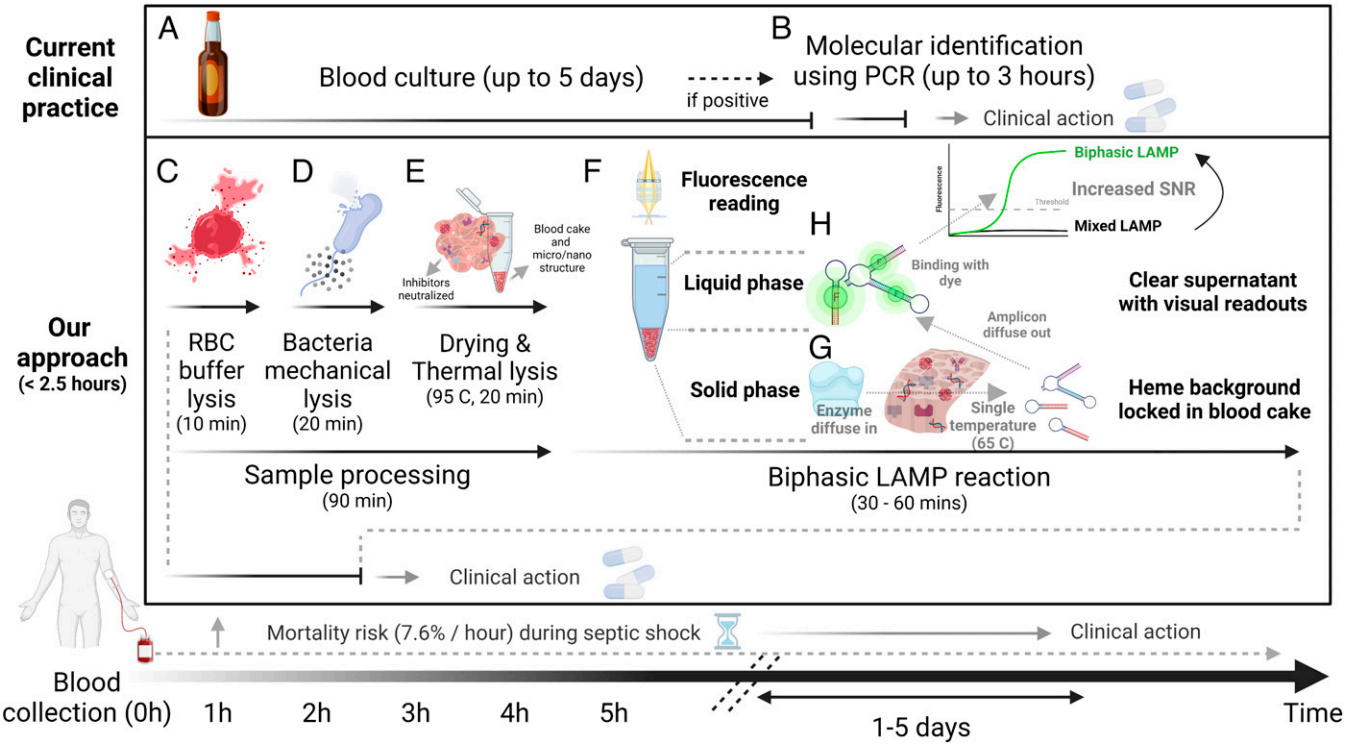


Fig. 1. Biphasic reaction for pathogen identification. (*A* and *B*) Blood culture–based PCR methods as current gold standard. (*A*) Diagnosis time is governed by blood culture time. (*B*) If the blood culture is positive, PCR is performed. (*C*–*H*) Protocol workflow of our blood-processing module and following biphasic LAMP reaction for culture-free pathogen identification. (*C*) RBC lysis using ACK lysis buffer. (*D*) Mechanical vortex for bacteria lysis and DNA extraction. (*E*) Direct drying of whole blood without purification to create dried blood matrix while inactivating the inhibitory elements in whole blood. Thermal lysis improves the porosity of microfluidic and nanofluidic networks within the dried blood matrix. (*F*–*H*) Biphasic LAMP reaction (*F*) where the solid phase (*G*) of dried blood matrix acts as a substrate. The enzyme initiates LAMP amplification at a single temperature (65 °C). The amplicons diffuse out to the liquid phase (*H*) and bind to the fluorescent dye in clear supernatant, increasing the signal-to-noise ratio (SNR). Total turnaround time is 2.5 h, including 1.5 h of sample processing and 1 h of LAMP reaction.

any other medical intervention (18–21). Specifically, a fivefold reduction in survival has been shown due to inappropriate antimicrobial therapy within the first 6 h of recognition (12). Apart from the long time to results, blood culture also suffers from other well-documented problems, such as suboptimal sensitivity (22), failure to identify slow-growing pathogens, and substantial delay or failure to identify pathogens in BSIs for patients who have previously received antibiotics (23). Pathogen detection from blood culture is worse in the neonatal patient population due to the limited sample volume (1 mL), with detection only possible in 10 to 15% of symptomatic neonates after excluding contaminants (24, 25). Given these limitations and challenges of blood culture, there is a need to develop analytical and molecular diagnostic approaches with faster time to results and better sensitivity.

Currently in the United States, nearly all US Food and Drug Administration (FDA)–approved sepsis molecular diagnostic platforms require blood culture as a first step and thus do not effectively improve patient management (26–29) (Fig. 1 *A* and *B*). Commercial kits performing nucleic acid amplification testing (NAAT) typically perform a separate, upstream purification step in which DNA and RNA from crude samples are extracted and purified using solid-phase extraction columns made from silica (30–32). Although the adsorption strength and capacity of these silica columns have been well characterized in previous works, most studies were conducted within the confines of high DNA loads, where the total input DNA exceeded 1 μg (33). In clinical applications, the workflow typically involves treating a biological sample (blood, urine, cerebral spinal fluid, etc.) with lysis buffer to release the nucleic acids from cells, bacteria, and/or virions. The DNA is then isolated from solution using a solid-phase extraction column, retrieved using an elution buffer, and quantified via molecular tests for diagnosis (33). This nucleic acid purification has two inherent loss mechanisms. First, DNA adsorption onto the column may be inefficient, and second, the purified DNA may not be efficiently eluted from the column (33). Hence, when processing ∼1 mL of blood, currently available nucleic acid purification kits cannot efficiently capture and retain these low-abundance copies of target pathogenic DNA against a vast background of contaminants and millions of copies of human genomic DNA. This is also the reason why the FDA-approved sepsis NAATs, such as Biofire's FilmArray and Nanosphere's Verigene, can only test for pathogens after a positive blood culture (Fig. 1 *A* and *B*) (34, 35). Of the few tests that circumvent the need for blood culture, most use conventional techniques such as PCR on purified DNA from whole blood or serum. Consequently, these tests suffer from low and variable sensitivity between 13 and 100% and an overall lower detection limit due to combined inefficiency of nucleic acid extraction and inefficiencies of downstream test-specific processes, such as splitting the extracted DNA into multiple reactions (1, 36–39).

Currently, there is only one FDA-approved pathogen identification platform from T2 Biosystems for BSIs. The T2 Biosystems' bacteria and *Candida* panels can identify specific pathogens from whole-blood samples in 5.4 h (mean time) (22), where the T2 bacteria panel was approved in 2018 (40). Although this technique bypasses the need for conventional DNA purification by using a proprietary mutant polymerase for performing pathogen-specific PCR from whole-blood lysate, it uses expensive reagents and instruments, such as magnetic nanoparticles and a magnetic resonance reader along with a thermocycler for PCR, because

visual readouts are impaired due to high heme background in the lysate. This increases the cost per assay and prevents possible translation into low- and middle-income countries. Moreover, the detection limit for *Escherichia coli* for the T2 bacteria panel is 11 colony-forming units (CFU)/mL, which falls short of the required sensitivity, especially for neonatal patients where studies have shown that concentrations in 68% of culture-positive cases fall well below 10 CFU/mL (41, 42).

To address the above challenges, we have taken a materials approach to whole-blood processing, which minimizes sample preparation and simultaneously offers unprecedented sensitivity. Here, we introduce a blood-processing module where we create a porous microfluidic and nanofluidic network within dried blood matrix (Fig. 1 *C*–*E*), allowing the polymerase to access the DNA inside the blood matrix and initiate amplification (Fig. 1 *F* and *G*). Previous studies have tried extracting viral nucleic acids using conventional purification techniques from dried blood spots on filter paper (limited to 50 μL of blood) but have showed amplification with limited sensitivity (43–45). Our continuum-scale simulation studies revealed that for low-CFU pathogen counts, the ideal approach would be to introduce the enzymes into the dried blood matrix through diffusion in microfluidic and nanofluidic networks instead of trying to elute the target DNA out of the blood matrix (Fig. 1 *E* and *G*). In our platform, the dried blood does not take part in the reaction and acts as a substrate through the duration of the reaction where the inhibitory elements, such as platelets, cells, and proteins, are neutralized and become a part of the substrate (Fig. 1 *E* and *G*). We show that the generated porosity and the microfluidic and nanofluidic network allow for enzymes to access DNA in the liquid phase and initiate amplification with single-molecule sensitivity inside the dried blood matrix, thus bypassing the need for conventional DNA purification (46) (Fig. 1*G*). The drying of blood can be accomplished in as low as 10 min at high temperatures (95 °C), significantly reducing the sample preparation time (Fig. 1*E*). Moreover, the dried blood solid phase does not re-mix with the supernatant and keeps the high heme locked in the background in red blood cells (RBCs) (Fig. 1 *F* and *G*), whereas the fluorescent amplicons after amplification are concentrated in the clear supernatant phase, giving an extraordinary signal to noise and fluorescence change even with more than 20% blood per reaction volume (Fig. 1*H*). Hence, we coined the term "biphasic amplification" for our reactions (Fig. 1 *F*–*H*). We couple this biphasic blood-processing module with a robust Bst polymerase, which we have previously shown to perform amplification in tissue matrices and loop-mediated isothermal amplification (LAMP) reaction, minimizing the need for a thermocycler (47). We first demonstrate our platform by efficiently amplifying cell-free methicillin-resistant *Staphylococcus aureus* (MRSA) and *E. coli* DNA in microliters of dried whole blood with single-molecule sensitivity (1 copy/4 μL of blood). We then couple our blood-processing module with mechanical bead lysis (Fig. 1*D*) to demonstrate a detection limit of 1.2 CFU/mL for MRSA (gram-positive), methicillin-sensitive *S. aureus* (MSSA; gram-positive), *E. coli* (gram-negative), and *Candida albicans* (fungus) pathogens from 0.8 mL of spiked healthy human blood samples. We tested 170 spiked samples, with 80 samples having concentrations below 10 CFU/mL. The reliability of the developed approach was further confirmed by testing 63 whole-blood clinical samples, including 14 positive for *E. coli* and 1 positive for MSSA (100% sensitivity and 100% specificity). The reliability of the developed approach was further confirmed by testing 63 clinical whole-blood samples, including 14 *E. coli*–positive, 1 MSSA-positive, and 15 culture-negative samples (100% sensitivity and 100% specificity). In addition, 40 samples culture positive for organisms other than MSSA, MRSA, *E. coli*, or *Candida* were also tested as specificity controls. The sample-to-answer time of our platform is less than 2.5 h. It is important to note that for *E. coli*, our platform is almost an order of magnitude more sensitive than the only currently FDA-approved culture-free bacteria panel (22).

Assay Design for Cell-Free DNA in Blood. We first designed the biphasic amplification process using spiked DNA in whole blood akin to cell-free DNA in whole blood in small-blood–volume reactions by adding 4 μL of whole blood with spiked pathogen DNA into standard 0.2-mL PCR tubes, followed by rapid drying of the blood in a heater (37 °C for 20 min) (Fig. 2*A*). This protocol represents the same process in Fig. 1 *E*–*H*. Scanning electron microscopy (SEM) images show that after drying, the blood becomes a solid substrate/sheet with a porosity at or below 6.4% (Fig. 2*B* and *SI Appendix*, Fig. S1). In the next step, we add the amplification buffer and reagents (without primers and polymerase) and subsequently generate a porous physical network inside the dried blood matrix by performing a wet thermal lysis for 95 °C for 2 min. The SEM characterization of dried blood after thermal lysis at different temperatures from 65 °C to 95 °C shows micronano-scale pores and networks. Image analyses show an increase in porosity of the dried blood matrix from ∼10% at the 65 °C lysis temperature to over 60% at 95 °C (Fig. 2 *C* and *D*). Hence, 95 °C was chosen as the final thermal lysis temperature. Physical microfluidic and nanofluidic networks can be seen inside the dried blood matrix after the thermal lysis step (Fig. 2*C* and *SI Appendix*, Fig. S1).

In addition to varying the blood drying conditions and thermal lysis times for porous network generation discussed above, we also explored the effect of increased thermal lysis times on the porosity of the dried matrix. To maximize porosity, we tried longer times of thermal lysis at 95 °C from 5 min to up to 20 min, and the SEM analysis of porosity is shown in *SI Appendix*, Fig. S2. As can be seen from the analysis, for whole blood, increased thermal lysis times gave similar porosity results (ranging between 63.61% and 65.24%) in comparison to what we previously observed with thermal lysis at 95 °C for 2 min. Hence, for our final protocol, we decided to use only 2 min of thermal lysis at 95 °C.

In the final step, the primers and polymerase were added, and the LAMP reaction was performed at a constant temperature of 65 °C for 60 min. This nanofluidic network allows primers and polymerase to access the DNA molecule via diffusion and initiate the amplification reaction inside the blood matrix. It is important to note that we use Bst polymerase for our biphasic reactions, which we have previously shown to be robust against tissue matrices (47). The solid dried blood phase also allows for a clear supernatant phase, where high signal to noise and a large fluorescence change can be observed during amplification, comparable to that of purified DNA reactions with no blood (Fig. 1 *F* and *G*). Because the optical reading components are located on the top of the reaction tube in the QuantStudio 3 system that we used, the solid dried blood at the bottom of the tube does not significantly interfere with the fluorescence reading from the fluid above the solid phase. Numerical simulation and experimental validation of the biphasic reaction mechanism can be found in *SI Appendix*, Results 1.

Detection of MRSA and *E. coli* Cell-Free DNA in Whole Blood in Biphasic Format. To evaluate the range and LOD of our biphasic assay for cell-free DNA, we next spiked serial dilutions

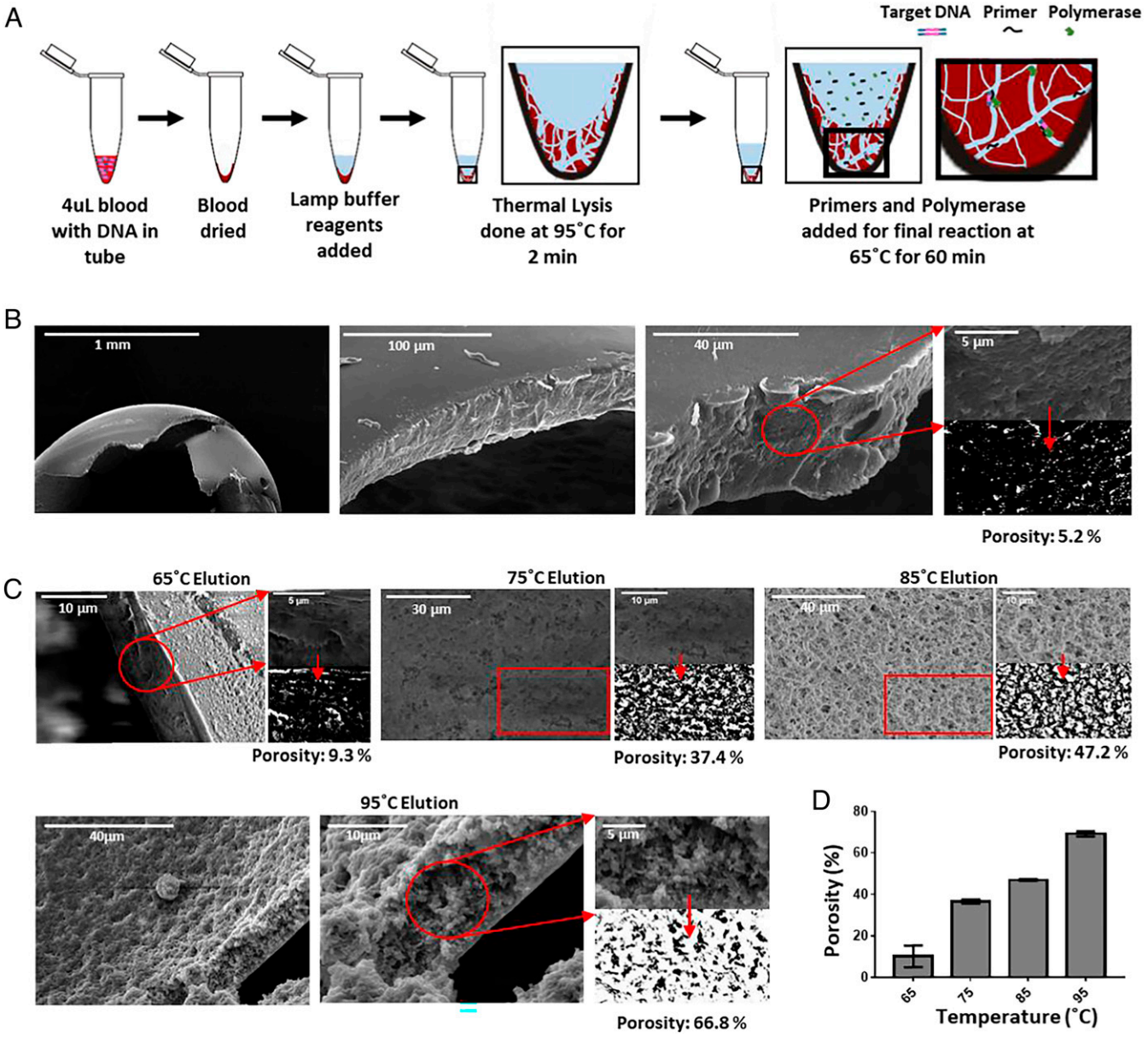


Fig. 2. Biphasic reaction schematic and analysis of blood before and after thermal lysis. (*A*) Process flow schematic of biphasic reaction. After drying, LAMP buffer reagents are added, and thermal lysis is conducted. Finally, primers and polymerase are added for the final reaction. Micro/nanofluidic channels are created during the thermal lysis heating step, so primers and polymerase may enter the blood matrix and find target DNA. (*B*) SEM images of the blood cake before thermal lysis. Image segmentation data show that the porosity of the blood cake is 5.2%. (*C*) SEM images of the blood cake after thermal lysis. Highest porosity is seen at 95 °C (66.8%). (*D*) Bars graph of the dried blood cake porosity versus thermal lysis temperature ($n = 3$ samples).

of MRSA and *E. coli* DNA in whole blood. For MRSA, we amplified the mecA gene, which is responsible for methicillin drug resistance. For *E. coli*, using previously published LAMP primers (48, 49), we amplified the malB gene, which is conserved in the majority of infectious *E. coli* strains.

First, to experimentally examine if the simple drying step and dried blood matrix provide enough sensitivity for the detection of cell-free DNA, we performed no thermal lysis controls. The porosity simulation (Fig. 3*A*) showed that the enzyme could not diffuse to the target DNA inside the blood matrix due to low porosity (~5%). The amplification curves and the threshold time bar graphs in no thermal lysis control reactions from blood are shown for MRSA DNA (*SI Appendix*, Fig. S5*A* and Fig. 3*B*) and *E. coli* DNA (*SI Appendix*, Fig. S5*B* and Fig. 3*C*). As predicted by porosity simulation, the detection limits for both the reactions were found to be 100 copies/4 µL of blood, highlighting the need for thermal lysis. Comparatively, it was shown that the extra thermal lysis step and consequent high porosity in biphasic reactions allows the enzyme to reach the DNA inside the blood matrix (Fig. 3*A*). The amplification threshold times in blood using our biphasic format are shown for MRSA DNA (Fig. 3*D*) and *E. coli* DNA (Fig. 3*E*). The amplification curves are shown in the *SI Appendix*, Fig. S5 *C* and *D*. The LOD for both the cases was found to be 1 copy/4 µL of whole blood (LOD is 1 copy because amplification frequency is equal to the expected sampling frequency), showing single-molecule sensitivity in our biphasic reactions. It is important to note that the created microfluidic and nanofluidic network allows access to even a single copy of DNA inside the solid blood matrix phase in our protocol. As expected, a larger range of amplification threshold times (10 to 20 min) was observed for low-DNA–copy number amplifications. Additional characterization of biphasic reactions with cell-free DNA can be found in *SI Appendix*, Results 2.

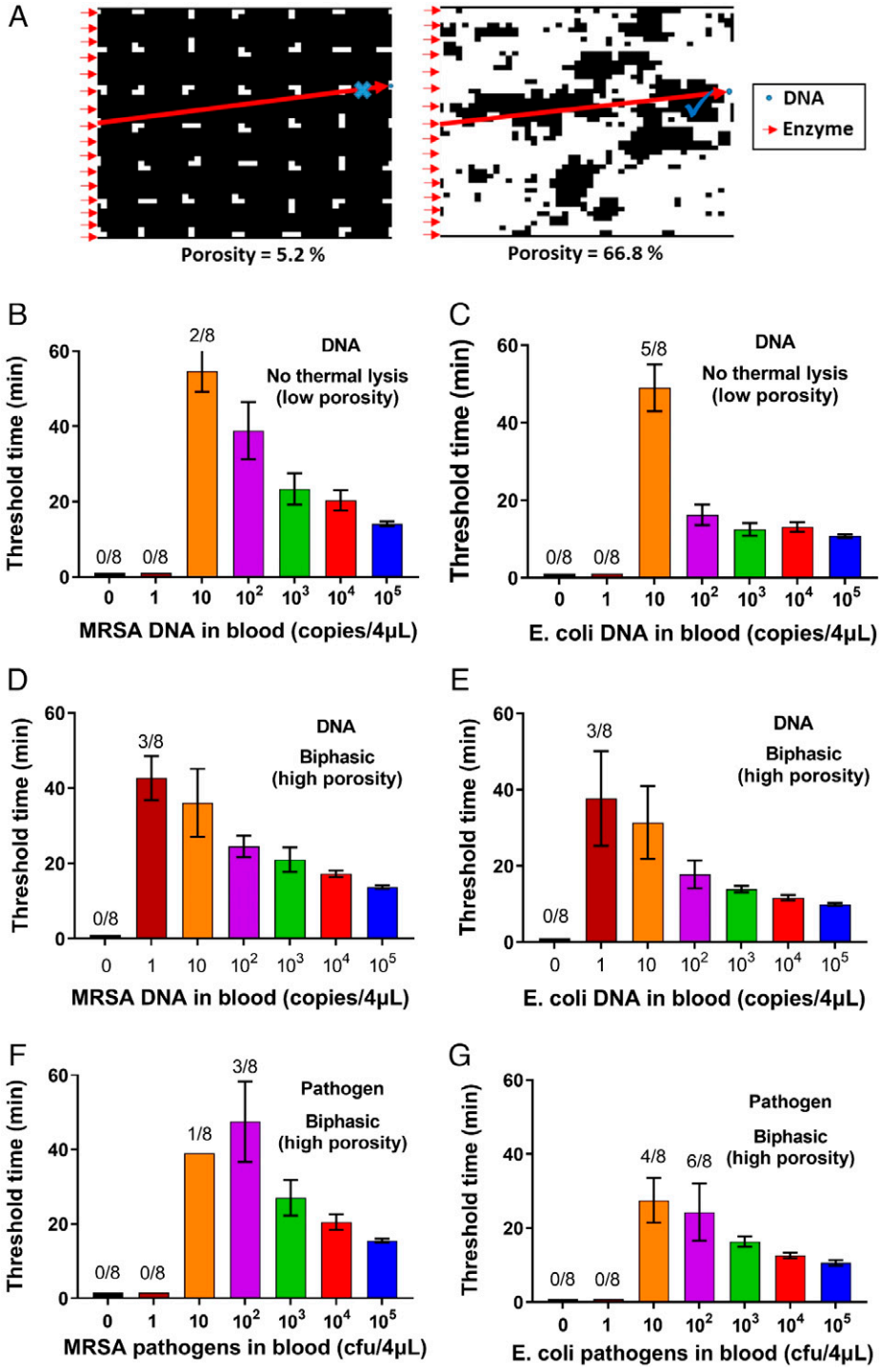


Fig. 3. Characterization of biphasic LAMP reaction with and without thermal lysis and biphasic LAMP reaction with pathogen lysis in whole blood. (*A*) Simulation of porosity differences before (~5%) and after (~67%) thermal lysis at 95 °C. (*B* and *C*) Single-molecule detection of MRSA and *E. coli* DNA in no thermal lysis control (low-porosity) reactions from whole blood. Amplification threshold timings for detecting MRSA (*B*) and *E. coli* (*C*). (*D* and *E*) Amplification threshold times for MRSA (*D*) and *E. coli* (*E*) DNA detection in the biphasic reaction (high-porosity reactions). For one-copy amplifications, an expected three out of eight amplifications are seen within 60 min of reaction time due to Poisson sampling statistics. (*F* and *G*) Characterization of pathogen lysis in whole blood. Amplification threshold times of biphasic reactions with MRSA (*F*) and *E. coli* (*G*) pathogens in 4 μL of whole blood. The bar graphs show mean and SD data from $n = 8$ replicates of amplification.

Detection of Low-CFU Pathogens in Whole Blood in Biphasic Format. Next, to translate our blood-processing and biphasic reaction module to detect pathogens in blood, we first carried out buffer reactions with pathogens spiked in phosphate-buffered saline (PBS) instead of blood. The amplification curves and the threshold times for MRSA (*SI Appendix*, Fig. S10 *A* and *B*) and *E. coli* (*SI Appendix*, Fig. S10 *C* and *D*) pathogens are shown. The LOD for both the pathogens was found to be 100 CFU, with only three of eight (MRSA) and two of eight (*E. coli*) replicates giving amplification for 10 CFU. This reduced sensitivity is expected, as thermal lysis (95 °C, 2 min), performed to disrupt the bacterial cell wall (*Materials and Methods*), has been previously shown to be inefficient in lysing bacteria (50, 51). Next, we repeated the above experiments with pathogens in blood in the biphasic reaction format for MRSA (*SI Appendix*, Fig. S10*E* and Fig. 3*F*) and *E. coli* (*SI Appendix*,

Fig. S10*F* and Fig. 3*G*). A similar reduced LOD of 1,000 CFU was observed for both pathogens, with only three of eight (MRSA) and six of eight (*E. coli*) replicates giving amplification for 100 CFU. These results highlight the need for coupling of our biphasic technique with a more efficient mechanical bacterial cell lysis approach to allow access to DNA and achieve improved sensitivity of our assay for detection of pathogens at low concentrations relevant to BSI and sepsis (52–55) (Fig. 1*D*). However, it is important to note that the optimized biphasic approach with small volumes of whole blood and moderate limits of detection is important in itself, for example, for finger pricks or heel lance nucleic acid testing in newborn blood samples (56).

Assay Design for ~1 CFU/mL LOD of Bacteria in Whole Blood. To address the challenges in pathogen identification in BSIs, specifically in sepsis where the pathogen concentrations can often be below 10 CFU/mL (41, 42), we coupled our biphasic blood-processing and reaction module with conventional bead-based mechanical pathogen lysis. We developed a protocol (Fig. 4*A*) where 800 µL of whole blood with pathogens is loaded into a 2-mL tube containing hypotonic RBC lysis buffer and 100-µm glass beads. The blood is mixed with the RBC lysis buffer to lyse the majority of the RBCs and centrifuged thereafter to pellet the intact cells. After discarding the supernatant from the RBC lysis, Tris-EDTA (TE) buffer is added, and mechanical bead lysis is performed by vortexing at 3,000 rpm for 10 min. Note that any cell-free DNA will also be discarded with the supernatant in the above step, and only intact cells will be retained. The blood lysate after mechanical bead lysis from a single sample is aliquoted into eight standard 0.2-mL PCR tubes with 30 µL per tube and dried for the biphasic amplification. The sample is considered positive for the target if any of these eight tubes (from the same starting sample) show amplification. The drying is performed by heating the sample at 95 °C for 10 min, followed by the LAMP reaction protocol for biphasic format (*Materials and Methods*).

To characterize the microenvironment, we performed SEM analysis of dried blood lysate after bead beating and found the porosity of the dried matrix before and after thermal lysis to be 11.5% and 63.8%, respectively, which is very similar to what we previously observed without bead beating. The SEM analysis can be found in *SI Appendix*, Fig. S11. It is important to note that for high-volume (0.8 to 1 mL) blood processing, we were able to rapidly dry 30 µL of blood lysate after bead beating at 95 °C while retaining the higher porosity after thermal lysis (~63.8%). This is likely because the clotting proteins and factors were removed along with the supernatant during the RBC lysis steps, while intact cells and pathogens were sedimented during centrifugation (6,000 × g, 10 min).

The threshold time bar graphs for MRSA (Fig. 4*B*), MSSA (Fig. 4*C*), and *E. coli* (Fig. 4*D*) spiked in 800 µL of whole blood are shown. The amplification curves for MRSA, MSSA

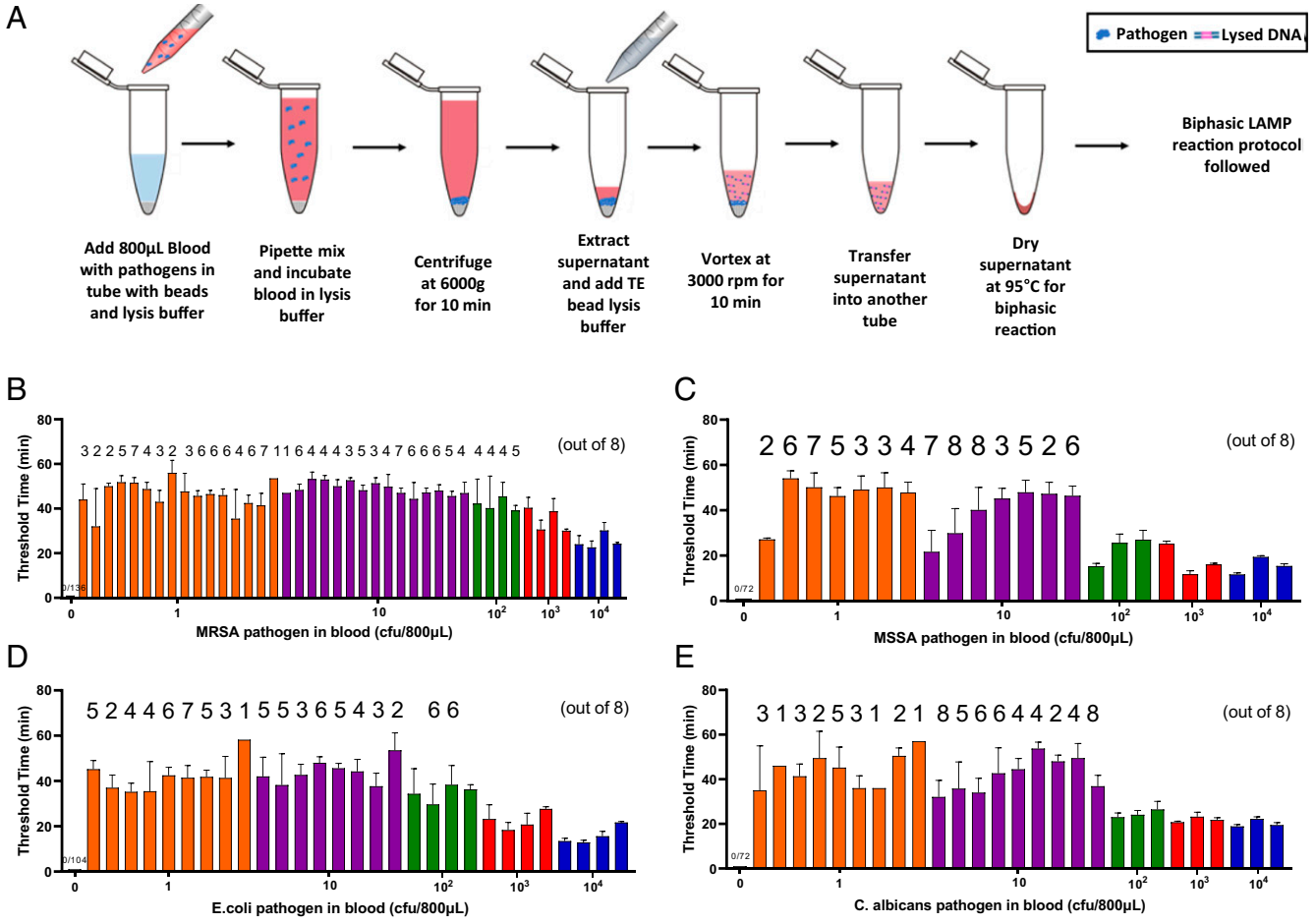


Fig. 4. Biphasic reaction coupled with mechanical pathogen lysis by bead beating for a detection limit of ~1 CFU/mL for MRSA, MSSA, *E. coli*, and *C. albicans*. (*A*) Process flow schematic consisting of RBC lysis, mechanical bead lysis, drying, and biphasic reaction from whole blood. (*B*–*E*) Amplification threshold data for the detection of MRSA (*B*), MSSA (*C*), *E. coli* (*D*), and *C. albicans* (*E*) pathogens in 800 µL of whole blood (eight curves for the eight tubes per 800 µL of starting blood sample). If not all eight tubes amplified for a sample, the number of tubes that amplified is indicated above. One bar represents one sample of 800 µL of whole blood spiked with a specific CFU count (1×10^4 to 1 or 0).

(fem A gene), and *E. coli* are shown in *SI Appendix*, Fig. S13 (48). It is important to note that the concentration range of the assay (1.2×10^4 to 1.2 CFU/mL) was chosen to overlap with the reported pathogen concentration in patients with BSIs (52–55). Moreover, MRSA, MSSA, and *E. coli* serve as good targets to demonstrate our platform not only because MRSA and MSSA are gram-positive (thicker cell wall) and *E. coli* is gram-negative, thus covering a range of bacterial infectious pathogens, but also because they have among the highest disease burden of all BSI pathogens (57). Overall, the detection of MRSA, MSSA, and *E. coli* was performed from 134 mock samples, where 62 samples were at 10 CFU or 1 CFU per 800 µL of whole blood, and 39 were negative-control samples (Fig. 4 and *SI Appendix*, Fig. S12). The LOD of our MRSA, MSSA, and *E. coli* assays in our platform was found to be 1.2 CFU/mL. While many more replicates need to be performed, we clearly show an improvement of an order of magnitude over the current state-of-the-art *E. coli* detection limit of 11 CFU/mL in the only FDA-approved blood culture–free diagnostic platform (22). Moreover, to confirm that our primers can distinguish between MRSA and MSSA, specificity tests were performed for MSSA primers using MRSA pathogens (*SI Appendix*, Fig. S13*A*) and MRSA primers using MSSA pathogens (*SI Appendix*, Fig. S13*B*). As a result, by using the MRSA and MSSA primers, the presence or absence of fem A and mec A genes (resistance genes) was identified, and therefore MRSA and MSSA could be distinguished using our platform. Control experiments and analysis of heme content for biphasic reaction can be found in *SI Appendix*, Results 3.

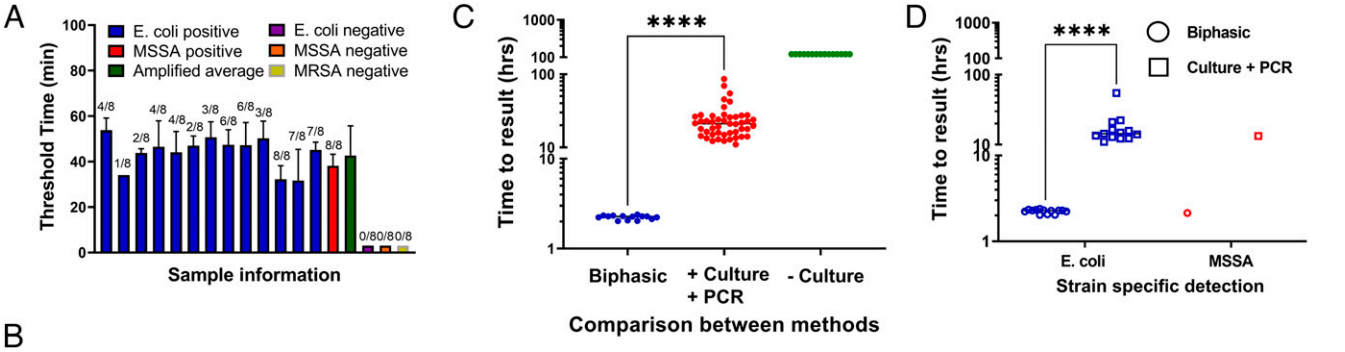
Assay Design for ~1 CFU/mL LOD of Fungal Pathogens in Whole Blood. To show that our platform applies to a broader pathogen range, we evaluated the LOD of fungal pathogens using mechanical bead lysis coupled with biphasic blood processing. Candidemia is a high-mortality (40%) fungal BSI caused by the *Candida* species of fungus where rapid diagnosis is crucial (12–14). Studies have shown that initiation of correct antifungal treatment in less than 12 h can reduce mortality from 40 to 11% (13, 53). However, its current clinical gold standard of diagnosis is blood culture, which takes 2 to 5 d for culture growth and has a low sensitivity of ~50% (58). Within *Candida* species, we chose to detect *C. albicans* in our platform because it is one of the most prevalent and is responsible for invasive candidiasis in the majority of the cases in the United States (59).

In comparison to bacteria, fungi are larger in size (10 to 12 µm), and their cell wall composition does not include peptidoglycan and lipid layers and instead includes layers of complex polysaccharides, including chitin, β-1,3-glucans, and β-1,6-glucans with cell wall proteins covalently bonded to this network (60, 61). This makes the fungal cell wall mechanically very strong and difficult to break. To disrupt the fungal cell wall, we modified our mechanical bead lysis to include larger 500-µm diameter glass beads (62), while the rest of the protocol remained the same. For the LAMP reaction, we targeted the intervening transcribed spacer 2 (ITS2) region within the *Candida* ribosomal DNA (rDNA) using previously published LAMP primers (63). We first optimized the reaction temperature and primer concentrations for our 800-µL high blood–volume biphasic format using the above primers. We found that a higher reaction temperature of 67 °C along with reduced primer concentrations of 0.04 µM F3 and B3, 0.33 µM FIP and BIP, and 0.17 µM LF and LB primers yielded the best results with no nonspecific amplification (*SI Appendix*, Fig. S17). The LOD experiments with the optimized protocol starting from 800 µL of whole blood spiked with *C. albicans* are shown (Fig. 4*E* and *SI Appendix*, Fig. S18). We could reliably detect 1 CFU/800 µL (LOD of 1.2 CFU/mL). Together, these figures show detection of *C. albicans* from 36, 800 µL of spiked whole-blood samples, with 18 of these being low-count samples (10 CFU, 1 CFU per 800 µL of blood) and 9 negative-control samples.

Assay Validation for Pathogen Identification from Clinical Whole-Blood Samples. Finally, we demonstrate the efficacy of our biphasic reaction to identify circulating pathogens in blood from clinical whole-blood samples using the process currently followed in clinical practice as a control. From February 2022 to April 2022, we collected a total of 724 samples, of which 63 samples (15 negative and 48 positive) were tested using our biphasic approach. The clinical samples were first analyzed by the Carle Foundation Hospital using current clinical practice (blood culture and PCR; Fig. 1 *A* and *B*), and the obtained results were compared with our results (biphasic approach). Protocol details for both analyses can be found in *Materials and Methods*.

Clinical laboratory results (including culture time and identification time) are summarized along with results from our biphasic process (test primers and threshold time) in *SI Appendix*, Fig. S19. To analyze the clinical samples, three primer sets (specific against *E. coli*, MRSA, and MSSA) were used. Of the clinical samples we tested, 14/63 samples (13 *E. coli* and 1 MSSA) were specific to targets that our primer sets can detect. Of these 14, 5 samples (sample ID numbers 46, 47, 48, 52, and 53) were tested with more than one set of primers to confirm specific identification and assay specificity (*SI Appendix*, Fig. S19). As a result, Fig. 5*A* demonstrates the threshold times for 14 amplified samples. The average threshold time for the 14 amplified samples was 42.5 ± 10.1 min. Considering that mock samples of *E. coli* and MSSA (>100 CFU/mL) were amplified within 40 min (Fig. 4 *C* and *D*), it can be inferred that most of the samples analyzed were <100 CFU/mL. However, no amplification was observed in the analysis of negative samples (15) nor during the analysis of positive samples (40) for organisms other than *E. coli*, MSSA, and MRSA. Fig. 5*B* summarizes the sensitivity and specificity of our assay. Our assay correctly identified all samples positive for *E. coli* and MSSA and identified all samples negative or positive for other organisms as negative for *E. coli*, MSSA, and MRSA, resulting in a sensitivity and specificity of 100%. These results, combined with the detection limit of 1.2 CFU/mL for the three target bacteria (confirmed by 134 mock samples), highlight the reliability of our biphasic assay, which avoids the need for blood culture.

Next, we compared the pathogen identification time required by the biphasic assay with the identification time required in the clinical laboratory. Our biphasic assay has shown an average amplification time of 42.5 min (*SI Appendix*, Fig. S19). Adding this amplification time to the sample preparation time (90 min), the total time required for the identification of the bacteria using the biphasic assay was obtained. This total identification time was compared with the time needed in the clinical laboratory (time to positive culture plus identification by PCR). The overall (Fig. 5*C*) and species-specific (Fig. 5*D*) times to result are shown to highlight the advantage of the biphasic assay in terms of response time. On average, while the biphasic reaction required 2.2 h to achieve pathogen identification, the clinical laboratory required 23.2 h. The *t* test demonstrated a clear statistically significant difference between the results of the biphasic assay and the clinical practices ($P < 0.0001$; Fig. 5*C*). This same behavior



B

	Our result	Blood culture + Identification result					
Species	Biphasic result	BC+ ID+	BC+ ID -	BC-	Metric	Value, % (n/N)	95% CI
E. coli	POS	12	0	0	Sensitivity	(12/12)	-
	NEG	0	9	2	Specificity	(11/11)	-
MRSA	POS	0	0	0	Sensitivity	-	-
	NEG	0	11	13	Specificity	(24/24)	-
MSSA	POS	1	0	0	Sensitivity	(1/1)	-
	NEG	0	11	0	Specificity	(9/9)	-
Overall	POS	13	0	0	Sensitivity	100%, (13/13)	75.29% to 100%
	NEG	0	31	15	Specificity	100%, (46/46)	92.29% to 100%

Fig. 5. Evaluation of the biphasic approach using clinical samples. (*A*) Threshold times of biphasic reactions for 14 amplified positive samples (13 *E. coli* and 1 MSSA) and average time (42.5 ± 10.1 min, with green bar) and not amplified samples for negative samples. (*B*) Table summarizing sensitivity and specificity of the biphasic approach against blood culture and identification using PCR. (*C*) Overall time-to-result comparison between the biphasic and blood culture and identification. (*D*) Species-specific time-to-result comparison between biphasic (circle) and blood culture and identification (square) for *E. coli* (blue) and MSSA (red). A statistical comparison was performed; 95% CI, 95% confidence interval (CI).

can be observed when analyzing only the *E. coli* detection results (Fig. 5*D*).

Rapid and accurate identification of pathogens causing BSIs has remained a significant diagnostic challenge in healthcare, especially in conditions such as sepsis where pathogen concentrations in blood can be as low as 1 CFU/mL. Due to the lack of rapid tests, blood cultures have remained the gold standard in diagnosing BSIs even though they take up to 5 d to produce results. It has also been shown that correct initial choice of antibiotic therapy within 1 to 3 h from initial symptom-based sepsis recognition can significantly reduce mortality. There are only a few diagnostic platforms that bypass the need for blood culture, but most of these platforms suffer from low and variable sensitivities due to inefficiencies in required conventional nucleic acid purification prior to detection in these platforms. Our approach presented here provides an alternative to blood processing and blood-based diagnostics for BSIs, where we rapidly dry the blood with pathogen DNA to generate a dried blood matrix and then create a physical microfluidic and nanofluidic network inside this dried blood matrix. Through simulations and experiments, we show that this generated microfluidic and nanofluidic network directly allows the amplification enzymes and primers to diffuse into the dried blood matrix, access the pathogen DNA, and initiate amplification inside the dried blood matrix, precluding any need for conventional nucleic acid purification. Further studies should be performed to understand the mechanisms and confirm that the blood-drying protocol inactivates the inhibitors and keeps them in the solid phase, allowing the target nucleic acid amplification in the liquid phase. For example, measurement of heme or hemoglobin in the solid and liquid phase by enzyme-linked immunosorbent assay (ELISA) or mass spectroscopy as a function of time and temperature could shed light into this hypothesis. This gives an extraordinary signal to noise and fluorescence change in our reactions, which is comparable to purified DNA (no blood) reactions, even with more than 20% blood per reaction volume. This biphasic approach significantly lowers the time of analysis and reduces the associated instrumentation complexity and consumable costs (*SI Appendix*, Table S2). We demonstrate our platform and the biphasic reaction approach on MRSA and *E. coli* cell-free DNA in whole blood and show single-molecule reaction sensitivity (1 copy/4 μL of blood dried per reaction). For cell-free DNA, we also show that compared to our biphasic reaction protocol, mixed blood reactions and reactions from only plasma after blood fractionation can have as much as three orders of magnitude higher (or worse) LOD. It is important to note that the optimized biphasic approach with small volumes of whole blood and moderate limits of detection is important in itself, for example, for finger pricks or heel lance nucleic acid testing in newborn blood samples (56). To detect a broad spectrum of pathogens and target the clinically relevant concentration range in sepsis, we coupled our biphasic blood-processing platform with mechanical bead lysis to disrupt the thick bacterial and *Candida* cell walls and allow access to DNA. In this format, we processed 0.8 mL of blood and showed a detection limit of 1.2 CFU/mL of blood for MRSA, MSSA, *E. coli*, and *C. albicans*. For *E. coli*, this detection limit is one order of magnitude improvement over the current state-of-the-art *E. coli* detection limit of 11 CFU/mL in the only available FDA-approved blood culture–free diagnostic platform (22). Our platform's superior detection limit can have a major impact especially for neonatal patients, where studies have shown that concentrations in 68% of culture-positive cases fall below 10 CFU/mL (41, 42). By contrast, because our method does not include purification and isolation of bacterial DNA because we directly dry the blood/blood lysate, we are able to capture and retain the few bacterial pathogens within the blood matrix. This results in a higher sensitivity in our biphasic method, allowing detection of pathogens at low concentrations relevant to sepsis without culture (52–55). We validated the biphasic assay by testing 63 clinical whole-blood samples. As a result of this validation, our assay showed 100% agreement with clinical laboratory results in terms of sensitivity and specificity

(no false positives were reported). Importantly, the average identification time using the biphasic approach (2.2 h) was significantly shorter than the mean pathogen identification time in the clinical laboratory (23.2 h).

Moreover, the current platforms require instrumentation, such as a magnetic resonance reader and magnetic nanoparticles, for detection of targets after DNA amplification due to the high background from blood lysate and heme. By contrast, our platform only requires a centrifuge, a heater, a vortex, and a fluorescence reader for performing all the steps, including blood drying, the microfluidic and nanofluidic network generation, and the isothermal biphasic amplification reaction using robust and commercially available Bst polymerase. With minimum expertise, such as accurate pipetting and possible contamination avoidance, these instruments have the potential to be optimized in an automated manner to handle large volumes of samples (~5 mL).

We also demonstrate the capability to detect genetic markers for drug resistance in pathogens by detecting the mecA gene in MRSA, which is responsible for its methicillin drug resistance. The current sample-to-result time in our platform is 2.5 h, with the potential to go down to less than 2 h with some automation. Also, assay time can be further reduced by skipping the thermal lysis step because the drying-only protocol has shown the ~1 CFU/mL sensitivity. Our platform can easily be scaled to process 5 mL or more of blood to further improve the detection limit. Importantly, this platform can also be used to detect viral pathogens from whole blood where the option to culture the pathogen does not exist and rapid detection of 1 plaque-forming unit (PFU)/mL or lower is required. While we used vials for drying the blood and performing the reactions, cartridges akin to a "pixelated Petri-dish" with a larger area and shorter height to accommodate 5 mL can provide for a more efficient drying of the blood. The clear supernatant in our reaction can allow visual or cell phone camera–based read out of pathogen amplification in our platform. Finally, we believe our platform will easily integrate into the current clinical workflow and significantly reduce costs and time to diagnosis of BSIs while providing state-of-the-art sensitivity.

Materials and Methods

DNA and Bacteria. Genomic DNA of MRSA strain HFH-30106, NR-10320, was obtained through BEI Resources. Genomic DNA of *E. coli* (O157:H7), NR-4629, was obtained through BEI Resources. These genomic DNA vials were aliquoted and stored at −80 °C. Appropriate stock volumes were used either for direct experimentation or diluted to the right concentration in buffer or whole blood. For experiments using pathogenic bacteria, MRSA strain HFH-30106, NR-10192, MSSA strain MN8, HM-162, and *E. coli* (O157:H7), NR-4356, were obtained through BEI Resources. For experiments using pathogenic fungus, *C. albicans*, strain L26, NR-29445 was also obtained through BEI Resources. These bacterial and fungal glycerol stocks were stored at −80 °C.

Bacterial Culture. Media and agar plates were obtained from the Cell Media Facility at the University of Illinois Urbana–Champaign (UIUC). Tryptic soy broth and agar were used for MRSA culture, and Luria–Bertani broth and agar were used for *E. coli* culture. Bacteria were grown in their respective broths at 37 °C for 16 h overnight, after which PBS stocks were prepared. *C. albicans* pathogens were grown in yeast peptone dextrose broth at 30 °C for 16 h overnight, after which PBS stocks were prepared.

PBS stocks of pathogens were prepared in accordance with the work of Liao and Shollenberger (64). Briefly, for bacteria, 250 μL of the overnight culture was centrifuged at 5,000 × g for 10 min to create a bacterial pellet, after which the pellet was washed twice with 1× PBS. Finally, the bacterial pellet was diluted in 1 mL of PBS, which was aliquoted and kept at room temperature. Each PBS stock was not used for more than 4 d after culture. PBS dilutions were done of the stock to the correct concentration and plated to know the bacterial concentration in the stocks. Based on the counts, the correct dilutions of the bacterial stocks were made in 1× PBS buffer or blood for the experiments. For fungus, PBS stock was prepared as described above, and the 1-mL aliquot was kept at 4 °C; each stock was not used for more than 48 h after culture. PBS dilutions were done of the stock, and a hemocytometer was used to calculate the correct concentration of the pathogen, based on which the correct dilutions of the fungal stocks were made in 1× PBS buffer or blood for the experiments.

Blood Preparation and Drying. Whole venous blood samples were drawn with a syringe from healthy, consenting MRSA- and *E. coli*-negative adult volunteers; samples were later transferred to 6-mL BD Vacutainer K2 Ethylenediaminetetraacetic acid (EDTA) collection tubes. The tubes were stored in a sample rotisserie at 4 °C before using them for experiments.

Tenfold serial blood dilutions of DNA or bacterial stocks were done to achieve the correct concentration required for experimentation. The spiked blood was then distributed into 0.2-mL PCR tubes (4 μL in each tube). This blood was dried on a hot plate at 37 °C for 20 min.

For blood drying characterizations, we tested different drying conditions, which can be found in *SI Appendix*, Table S1. Samples of the dried blood before and after thermal lysis were prepared, and SEM analysis was performed to quantify the porosity of the blood matrices. The details of the blood drying temperature, time, and corresponding porosity before and after thermal lysis are summarized in *SI Appendix*, Table S1.

Primer Sequences. All primer sequences for the LAMP reactions were synthesized by Integrated DNA Technology. Primer sequences for the MRSA mec A gene were obtained from Xu et al., and sequences for the *E. coli* mal B gene were obtained from Hill et al. (48, 49). Primer sequences for the *C. albicans* ITS2 region were obtained from Kasahara et al. (63).

LAMP Reactions. The LAMP assay was designed to target the mecA gene for MRSA, the malB gene for *E. coli*, and the ITS2 region for *C. albicans*. The LAMP assay is comprised of the following components: 1× final concentration of the isothermal amplification buffer (New England Biolabs), 1.025 mmol L^{-1} each of dNTPs, 4 mmol L^{-1} $MgSO_4$ (New England Biolabs), and 0.29 mol L^{-1} betaine (Sigma-Aldrich). These individual components were stored according to the manufacturer's instructions, and a mix including all components was created fresh before each reaction. In addition to the buffer components, 0.15 μM F3 and B3, 1.17 μM FIP and BIP, and 0.59 μM LoopF and LoopB primers, 0.47 U μL^{-1} Bst 2.0 WarmStart DNA polymerase (New England Biolabs), 1 mg/mL bovine serum albumin (BSA; New England Biolabs), and 0.74× EvaGreen (Biotium), a double-stranded DNA intercalating dye, were included in the reaction. The final reaction volume was 16 μL. In case of buffer reactions, 1 μL of template in water or bacteria in 1× PBS buffer was added to make the final reaction volume 16 μL.

The format of biphasic blood reactions was as follows. In tubes with 4 μL of dried blood, 4 μL total of the buffer mix, BSA, and dye in the correct concentrations was added so that final reaction concentrations were as mentioned above. After thermal lysis, 12 μL total of buffer mix, BSA, dye, and primers and polymerase was added to make a final 16-μL reaction.

The format of mixed blood reactions with DNA-spiked whole blood was as follows. In tubes with 4 μL of spiked blood, 16 μL total of the LAMP reaction reagents, including primers and polymerase in the final concentrations mentioned above, was added and mixed. For mixed reactions with supernatant of fractionated blood, 100 μL of DNA-spiked blood was centrifuged at 5,000 × g for 10 min, and supernatant with DNA was extracted and distributed in 4-μL aliquots into tubes. Thereafter, the 16 μL of LAMP reaction reagents was added in the final concentrations mentioned above and mixed.

All the LAMP tests were carried out in 0.2-mL PCR tubes in an Eppendorf Mastercycler realplex real-time PCR system. The tubes were incubated at 65 °C for 60 min in the thermocycler, and fluorescence data were recorded every 1 min during the reaction. Eight replicates were done for each reaction.

Reaction in Blood Cake. Reactions discussed in *SI Appendix*, Fig. S3, conceptually showed that amplification starts within the porous channels of the blood cake. Experimentally, this was designed in the following format. In tubes with 4 μL of dried blood, 8 μL total of the buffer mix, BSA, and dye was added at the same final reaction concentrations mentioned above. After thermal lysis, 4 μL of

the supernatant was removed from the tubes. Then, 12 μL total of buffer mix, BSA, dye, and primers and polymerase was added to make the final reaction volume 16 μL.

High-Volume Blood Processing with Biphasic Reactions. For high-blood–volume reactions and effective pathogen lysis, a bead-beating protocol was used similar to that of T2 Biosystems (65). First, in a 1.5-mL tube, 800 μL (or 1 mL) of blood with the correct concentration of pathogens was added to a tube with 40 μL (90 mg) of glass disruptor beads (Scientific Industries, Inc.) and 600 μL of blood lysis buffer (consisting of 10 mmol L^{-1} $KHCO_3$, 150 mmol L^{-1} NH_4Cl, and 0.1 mmol L^{-1} EDTA).The blood and lysis buffer were manually pipetted and left to incubate at room temperature for 5 min. After centrifugation at 6,000 × g for 10 min, the lysed blood supernatant was removed, and 200 μL of TE buffer was added to the tube for bead lysis. The tubes were vortexed at 3,000 rpm for 10 min. Finally, after a brief 10-s centrifugation, the 30 μL of lysate was distributed from the 1.5-mL tubes into as many 0.2-mL PCR reaction tubes as necessary to extract the complete lysate. This aliquoted lysate was dried at 95 °C in a heater for 10 min, and the biphasic reaction protocol was followed thereafter. For these reactions, the format was as follows. In tubes with 30 μL of dried lysate, 72 μL of the buffer mix, BSA, and dye in the correct concentrations was added so that final reaction concentrations were as previously mentioned. After thermal lysis, 24 μL total of buffer mix, BSA, dye, and primers and polymerase was added to make a final 96-μL reaction. These assays were incubated at 65 °C for 60 min in the thermocycler, and fluorescence data were recorded every 1 min during the reaction.

For mixed blood reactions, in which blood lysate after blood processing and bead beating was not dried, the reaction format was as follows. In tubes with 30 μL of lysate, 66 μL of the LAMP reaction mix components, including primers and polymerase, was added in the correct concentrations so that the final 96-μL reaction concentrations were equivalent to those previously mentioned. The maximum recommended liquid capacity for PCR tubes is 100 μL. The assays were incubated at 65 °C for 60 min in the thermocycler, and data were collected.

For effective lysis of *C. albicans* fungal pathogens, the blood lysis and bead-beating protocol was followed as mentioned above but with 500-μm glass disruptor beads (Scientific Industries, Inc.). For the biphasic reaction done with dried blood lysate, the reaction composition was the same as that previously mentioned for the 96-μL reaction, and incubation for the amplification occurred at 62 °C for 60 min, which was later optimized to occur at 67 °C for increased specificity of the assay. These reactions were further optimized by decreasing the final concentration of each primer in the 96-μL reaction: 0.04 μM F3 and B3, 0.33 μM FIP and BIP, and 0.17 μM LoopF and LoopB.

Amplification Data Analysis. The off-chip raw fluorescence curves and amplification threshold bar graphs were analyzed using a MATLAB script and plotted using GraphPad Prism. The threshold time for each curve was taken as the time required for each curve to reach 10% of the total intensity. The amplification threshold bar graphs are show a mean of eight samples.

Clinical Samples. The clinical samples were discarded whole-blood samples from patients in the emergency department (ED) that had a blood culture ordered. Samples were collected at Carle Foundation Hospital (Urbana, IL) through an approved institutional review board study (Carle IRB 21BIO3462). The samples were transferred to UIUC and stored at 4 °C until use. The clinical procedure for pathogen identification can be seen in *SI Appendix*, Methods 1.

Data, Materials, and Software Availability. All study data are included in the article and *SI Appendix*.

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