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Mobile Platform for Multiplexed Detection and Differentiation of Disease-Specific Nucleic Acid Sequences, Using Microfluidic Loop-Mediated Isothermal Amplification and Smartphone Detection

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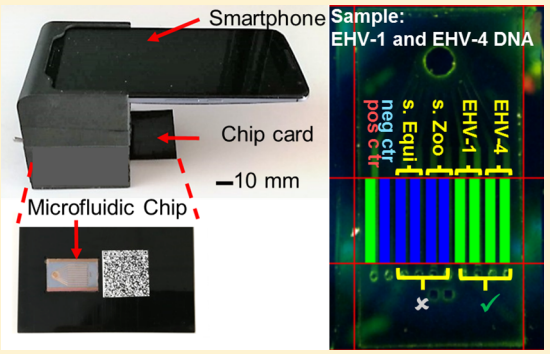
Supporting Information

ABSTRACT: New tools are needed to enable rapid detection, identification, and reporting of infectious viral and microbial pathogens in a wide variety of point-of-care applications that impact human and animal health. We report the design, construction, and characterization of a platform for multiplexed analysis of disease-specific DNA sequences that utilizes a smartphone camera as the sensor in conjunction with a hand-held "cradle" that interfaces the phone with a silicon-based microfluidic chip embedded within a credit-card-sized cartridge. Utilizing specific nucleic acid sequences for four equine respiratory pathogens as representative examples, we demonstrated the ability of the system to utilize a single 15 μL droplet of test sample to perform selective positive/negative determination of target sequences, including integrated experimental controls, in approximately 30 min. Our approach utilizes loop-mediated isothermal amplification (LAMP) reagents predeposited into distinct lanes of the microfluidic chip, which when exposed to target nucleic acid sequences from the test sample, generates fluorescent products that when excited by appropriately selected light emitting diodes (LEDs), are visualized and automatically analyzed by a software application running on the smartphone microprocessor. The system achieves detection limits comparable to those obtained by laboratory-based methods and instruments. Assay information is combined with the information from the cartridge and the patient to populate a cloud-based database for epidemiological reporting of test results.



Infectious diseases, such as HIV/AIDS, tuberculosis, and malaria, while accounting for less than 10% of deaths in the developed world, are responsible for more than half of all human deaths in developing countries.[1] The societal and economic toll of infectious disease is not limited only to human populations. Animals raised for human consumption live in facilities in which large herds share ventilation, feed, and waste handling within confined spaces in which respiratory infectious disease can spread rapidly. Similar concerns exist for companion animals and racing animals, particularly for those with high economic value.

Respiratory disease is common in the horse and difficult to diagnose using the current diagnostic methods available to veterinarians.[2] Infectious disease outbreaks can be financially devastating,[3,4] and thus there is a compelling need to provide veterinarians with the ability to perform point-of-care diagnostic tests of infectious disease in horses, food animals, and companion animals. Ideally, such a device would enable veterinarians to rapidly diagnose disease in their office or in the field, resulting in better-informed management decisions, while markedly improving the control of animal disease outbreaks. In this work, we report a portable battery operated diagnostic device, capable of diagnosing multiple pathogens simultaneously, using a disposable, one-time-use credit-card-format

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microfluidic chip that allows for viral or bacterial pathogens to be tested simultaneously with results obtained in approximately 30 min.

A key characteristic for a successful system for detection and reporting of infectious disease is speed. For example, modeling studies on various intervention strategies for pandemic influenza response have shown that the highest impact on attack rate is obtained by identification and initiation of treatment 1 day earlier.[5] Particularly for point-of-care scenarios, where the clinician is testing a patient at a remote clinic, a farm, or a racetrack, the need to send samples to a central laboratory, to wait for the test to be performed, and to wait for the results to be reported, results in an enormous waste of opportunity to determine if aggressive treatment or quarantine is needed before the disease spreads further. The ability to rapidly share the results of positive and negative tests can revolutionize the manner in which infectious diseases are managed. Therefore, it is of paramount importance for the test to be performed at the same location as the patient, so action can be taken within the same day that the sample is gathered.

Taking advantage of the fact that bacteria and viruses have distinct genetic components that are represented by unique nucleic acid sequences, the most commonly used laboratory analysis technique used for diagnosis of infectious disease is polymerase chain reaction (PCR).[6−8] Conventional PCR amplification requires expensive laboratory-based instruments that are operated by technicians and housed in central facilities, although there have been strong efforts aimed at miniaturization of PCR for translation closer to the point-of-care through the engineering of systems integrated into a small chip or cartridge.[6,8−10] Due to the cost and complexity of implementing thermal cycling, various isothermal nucleic acid amplification methods have been proposed and demonstrated with comparable sensitivity to PCR,[11−13] with a requirement to more fully customize and validate the primer sequences. Of these, loop-mediated isothermal amplification (LAMP) has emerged as a compelling approach for portable applications,[14−19] particularly due to its ability to utilize unprocessed or minimally processed test samples without inhibition of the reaction. Details of the LAMP process, primer design, and example applications have been thoroughly explained.[20]

An important prerequisite for the widespread adoption of point-of-care (POC) tests is the availability of detection instruments that are inexpensive, portable, and able to share data wirelessly over the Internet.[21−24] Due to the rapid development of computational, communication, and sensing capabilities of smartphones since the introduction of the iPhone in 2007, these devices have become similar to personal computers with integrated cameras, geolocation capabilities, and access to cloud services. Since 2011, over 478 million smartphones are sold annually, with that number expected to double in the next 4 years,[25] making them a nearly ubiquitous tool that can be adapted to performing POC tests. Recent examples include attachments that enable smartphones to serve as stethoscopes,[26] ultrasound probes,[27] microscopes,[28] fluorescent microscopes,[23,29] label-free biosensor detection instruments,[30,31] fluorimeters,[32] and colorimetric assay readers.[33] Portable detection systems for infectious disease are already recognized as a likely extension of mobile technology,[34−36] a PCR or LAMP mobile sensing platform that is integrated with a smartphone and a smart service system for reporting and sharing results with a network of users is highly desirable.

In this work, we have developed a smartphone-based portable detection instrument to perform isothermal LAMP-based analysis for the diagnosis of equine respiratory infectious diseases. While our ultimate goal is the diagnosis and mobile reporting of human infectious diseases, we initially focus specifically upon equine respiratory infections due to their economic importance to the horse racing industry, similarity to the tests that would have an impact upon the food animal industry, and the strong need for a service system that can inform networks of field veterinarians. Our work involves the development of a microfluidic approach for identifying the presence of specific nucleic acid sequences from the pathogens of equine respiratory infections using a portable detection system integrated as a disposable cartridge that can be read by a conventional smartphone in conjunction with a custom-designed cradle. Capable of simultaneously performing 10 parallel isothermal amplifications (with two utilized for experimental controls and eight for pathogen-specific DNA detection assays), the system can detect target DNA sequences from more than one pathogen with a single test protocol and interface with a smartphone app that communicates with a cloud-based service for the immediate reporting of the location, time, identity, and results (positive and negative) of the detection. In this work, four LAMP-based assays have been developed for the detection of four important vectors of equine infectious respiratory diseases: *Streptococcus equi* (S. Equi), *Streptococcus zooepidemicus* (S. Zoo), and *Equine herpesvirus* type 1 and type 4 (EHV-1 and EHV-4). The smartphone-based detection demonstrates the same detection limits and sensitivity as LAMP reactions performed on commercially available laboratory-based systems and is capable of identifying the specific nucleic acid sequences of pathogens for both single-infection and co-infection scenarios. To our knowledge, a smartphone-based nucleic acid testing approach for the diagnosis of veterinary infectious diseases has not been reported in previous literature.

Our approach utilizes a silicon microfluidic chip with LAMP primers applied to individual assay lanes, which is embedded within a credit-card-format plastic holder to facilitate handling and to carry assay-specific information. The chip accepts a single droplet (~15 μL) test sample that is distributed between 10 separate regions. After a ~30 min LAMP reaction (conducted on a hot plate, separate from the cradle), the plastic card is inserted into a slot in the body of a cradle (in the same manner as chip readers used for modern credit cards) that places the chip in contact with a heater from below, while illuminating with light emitting diodes (LEDs) from above. The cradle accepts an unmodified smartphone, placing the chip in front of its rear-facing camera, which gathers a fluorescent image of the assay lanes through a macrolens (to increase the field of view) and an optical emission filter. Image processing software operating on the smartphone automatically recognizes the regions of interest within the fluorescence image that correspond to the relevant assay lanes and determines a fluorescent intensity value for the positive control, negative control, and each assay. The system reports normalized fluorescent end point intensity values of each lane and generates a positive/negative determination for each assay in ~5 s. The software application operating on the smartphone gathers information about the tests conducted on the microfluidic card, patient-specific information, and results from the assays that are communicated to a cloud storage database. Although not the focus of the results presented here,

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the database anticipates queries from a network of geographically distributed users who seek updates when positive tests are recorded within user-defined constraints (such as geographic area, disease type, animal category, or date) and the ability to graphically represent trends such as temporal sequences of positive tests overlaid with location, and trends over time. We anticipate that a smart service system for clinicians would also facilitate messaging communication between users to easily share information such as specific circumstances of tests and the outcomes of treatment. Integration of a smartphone-based testing capability and smartphone-based epidemiology capability represents a future goal of the sensing capability reported here.

EXPERIMENTAL SECTION

Chip Substrate Fabrication. To perform multiplexed LAMP detection of pathogen-specific nucleic acids, a silicon-based microfluidic chip with 10 parallel flow channels and a shared sample inlet was designed and fabricated. The chip is the size (25 mm × 15 mm) of a standard SIM (subscriber identity module) card. Flow channel dimensions are 10 mm in length, 500 μm in width, and 200 μm in depth, representing a volume of 1 μL each. The inlet of the chip is a 4 mm diameter entrance chamber that feeds its contents to 10 parallel assay channels. Two square markers at the opposite ends of the chip are used for position alignment during fabrication and for automated image recognition during the assay measurement. The fabrication process for the microfluidic chip is described in the Supporting Information and illustrated in Figure 1a. The scanning electron micrograph (SEM) in Figure 1c shows the vertical sidewalls of the etched channels. After the Bosch process, the remaining photoresist was stripped with acetone and O_2 plasma cleaning, leaving the bare silicon exposed. Because bare silicon has been reported to have inhibitory effects on nucleic acid amplification due to absorption of polymerase,[8]

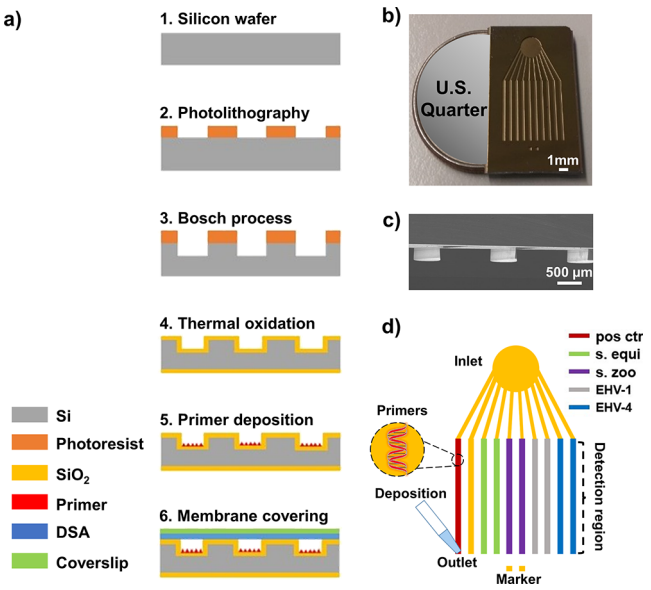

Figure 1. Ten flow channel microfluidic chip for multiplexed LAMP detection. (a) Schematic diagram of the fabrication process for the microfluidic chip. (b) Photograph of a fabricated chip taken with a U.S. quarter. (c) Scanning electron microscope (SEM) image of the cross-section of the microfluidic channels. (d) Deposition of the primers and positive control for the on-chip reactions.

the wafer was thermally oxidized in a furnace (1150 °C) for 2 h to grow a 200 nm film of SiO_2. The final step of the chip fabrication was to dice the wafer into individual chips (Figure 1b).

Deposition of the Primers and Positive Control. The primers are uniformly deposited onto the surfaces of the flow channels before sample loading through pipet injection at the outlet of each channel. Figure 1d shows the deposition operation for the four types of primers and positive control. The positive control lane is comprised of a primer and its matching template DNA to indicate that the microfluidic channels have been filled with fluid and that the thermal conditions required for the LAMP reaction have been provided to the chip. While we have chosen to use the primer and target DNA for our S. Zoo assay, in principle any LAMP primer/target pair could be utilized, such as those for housekeeping genes. Using a negative control with no primer enables determination of background fluorescent intensity with no self-fluorescence from primers, representing the lowest value obtainable.

Channel 1 serves as the positive control by depositing a mixture of the primer set and template DNAs for S. Zoo, while channel 2 serves as the negative control because no primers are deposited within it. The remaining eight channels are divided into four groups to allow for the deposition of four types of primer sets used in this work, with channels 3 and 4 prepared with primers for the S. Equi assay, channels 5 and 6 deposited with primers for the S. Zoo assay, channels 7 and 8 deposited with primers for the EHV-1 assay, and channels 9 and 10 deposited with primers for the EHV-4 assay. The primer solutions injected into the eight channels are prepared with a direct 20-fold dilution of the corresponding primer solution (originally at 55 μM) in nuclease-free water, resulting in a final primer concentration of 2.75 μM. For the solution injected in the positive control channel, 1 μL of the S. Zoo DNA sample solution at a concentration of 5×10^6 copies/mL is mixed with 1 μL of 20 times diluted 55 μM S. Zoo primer solution to make a 2 μL final solution. After the solutions are prepared, a volume of 1 μL of each solution is taken by a pipet and injected into the corresponding flow channels from the end of each channel. The injected liquids can reach the opposite ends of the flow channels without entering the common inlet hole due to their volumes, and all the primers completely dry on the channel surface at room temperature within a few minutes. The primer deposition process enables batches of chips to be prepared in advance and stored for later use. Following primer deposition, the microfluidic lanes are sealed with double-sided adhesive (DSA) and incorporated within a credit-card-shaped plastic holder that holds the chip and a quick response (QR) code that stores information about the assays within the chip, as described in Supporting Information.

Smartphone-Based Instrument. The smartphone-based instrument to measure the fluorescent emission from the on-chip LAMP reactions is shown in Figure 2. The system consists of a smartphone (Nexus 6; Motorola, IL, USA) and a 3D-printed plastic cradle body that contains the optical and electrical components to excite and collect fluorescence signals at a constant, controlled temperature. The top part of the cradle interfaces with the rear-facing camera (13 megapixels, pixel size of 1.4 μm). The cradle aligns the smartphone camera with the internal optomechanical components and also serves as a dark chamber for fluorescence detection by excluding external light. A schematic of the fluorescence imaging system is depicted in

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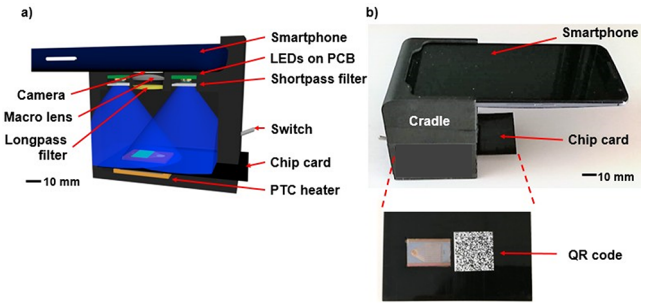

Figure 2. Smartphone-based instrument. (a) Schematic diagram to show the internal structure of the cradle that integrates optical and electrical components used for smartphone fluorescence microscopy. The microfluidic chip, integrated in its card, is inserted into the cradle that incorporates a PTC heater to maintain a constant ~65 °C temperature. (b) Photograph of the smartphone-based instrument taken with the smartphone and chip card. A QR code label is printed on the chip card to provide information about the on-chip detection.

Figure 2a. When the mobile phone is inserted into the slot at the top of the cradle, the rear-facing camera is aligned over a window (30 mm) that is formed by a 525 nm long pass filter (no. 84-744; Edmund Optics, NJ, USA)) and a 12.5× macrolens (no. TECHO-LENS-01, TECHO) in series. The long pass filter is selected according to the emission wavelength of the fluorescent dye (EvaGreen) used in the LAMP assay. The macrolens placed in front of the camera facilitates close-up photography of the chip, allowing the reduction of the distance between the camera and the chip to 50 mm while keeping the field of view as large as 24 mm × 24 mm with negligible barrel distortion. We designed and built a custom light source module comprised of eight 485 nm blue LEDs (no. XPEBBL; Cree Inc., NC, USA) and four 490 nm short pass filters (no. ZVS0510; Asashi Spectra, Tokyo, Japan) that cover each pair of LEDs installed at the upper corners of the cradle to provide excitation illumination that does not spectrally overlap with the dominant fluorescence emission wavelengths. The LEDs are mounted on a custom-built printed circuit board (PCB) and arranged with square symmetry to support uniform illumination over the whole chip area, using ray-tracing software (Zemax) to ensure <2% variation in illumination intensity over the active area of the chip. The light source module is powered by two AAA batteries and operated using an on/off control switch located on the cradle body. A positive temperature coefficient (PTC) heater (12 V-80 °C; Uxcell, Hong Kong, China) is placed beneath the chip allowing the chip to be maintained at a temperature of 65 °C without an additional temperature controller. The PTC heater is made from specific ceramic materials that have a highly nonlinear thermal response and a positive thermal coefficient of resistance. When the temperature of the heater exceeds a composition-dependent threshold, the electrical resistance increases resulting in decreased power output to set the temperature at a predefined limit. The PTC heater is powered by a standard 9 V battery to set the temperature of the microfluidic chip to 64−66 °C during the measurements. The heater is also operated with an on/off control switch. The overall dimensions of the cradle are ~90 mm × 70 mm × 95 mm. The purpose of the PTC heater is not to provide thermal energy for the LAMP reaction but to maintain the chip at an elevated temperature during fluorescence imaging, in which fluorescent background intensity is reduced compared to room temperature, which increases signal-to-background measurement of the LAMP assays. Thus, extended heating and precise temperature control are not required. The retail cost of the components used to construct the instrument, with components purchased individually, is approximately $550, and the system weight is approximately 15 ounces, not including the phone.

RESULTS

Detection of Target DNA Sequence in the Presence of Target and Nontarget Primers. The following experiments were designed to demonstrate the ability of this approach to detect viral and bacterial DNA in the presence of interfering nontarget DNA. DNA was extracted directly from the corresponding viral or bacterial stock by heat lysis. Briefly, 1 mL of culture-grown equine pathogens are centrifuged at 12,000 rpm for 1 min. The supernatant is discarded, and the remaining material is suspended in 200 uL of nuclease-free water. The sample is next heat-lysed at 95 °C for 5 min, followed by 3 min of centrifugation at 12,000 rpm. The supernatant containing the extracted pathogenic DNAs is retrieved and ready for immediate use or stored at −80 °C until use. Plasmids were designed and fabricated that contain the target nucleic acid sequences, to assist in quantitation of detection limits. The copy numbers of target DNA reported here represent estimates derived by using LAMP standard curves that were established using the synthetic plasmid targets of known concentrations (Figure S2), for which the threshold time for the plasmid concentration corresponds to the copy number of target nucleic acid derived from pathogen culture extract. The selectivity of the LAMP primers for their intended targets were validated using "off-chip" reactions performed in PCR tubes and measured with a laboratory detection instrument, as described in Supporting Information and shown in Figure S10 and Table S1. LAMP and PCR primer sequences for each target are detailed in Table S2. Using the threshold time of the reaction to quantify the limit of detection for the laboratory-based off-chip LAMP and PCR assays, we measured limits shown in Table 1 derived from the kinetic amplification plots shown in Figures S1 and S3 in the Supporting Information. Four types of primers used in the four LAMP assays and the positive control mixture were deposited within the microfluidic channels according to the method described in Deposition of the Primers and Positive Control. Here, we utilized eight assay wells to detect four different target DNA sequences, with two replicate lanes for each sequence. When pathogen-specific target DNA sequences are present in the sample injected into the microfluidic chip, the corresponding two channels as well as the positive control channel will generate an amplification reaction with associated fluorescent emission, while the other lanes remain dark. By measuring the fluorescence output from each channel using the

Table 1. Limit of Detection (LOD) Comparison of the Four Assays for Off-Chip PCR, Off-Chip LAMP, and On-Chip LAMP

target	off-chip PCR (copies/μL)	off-chip LAMP (copies/μL)	on-chip LAMP (copies/μL)
S. Equi	100	50	50
S. Zoo	100	5	50
EHV-1	100	5	5
EHV-4	100	1	5

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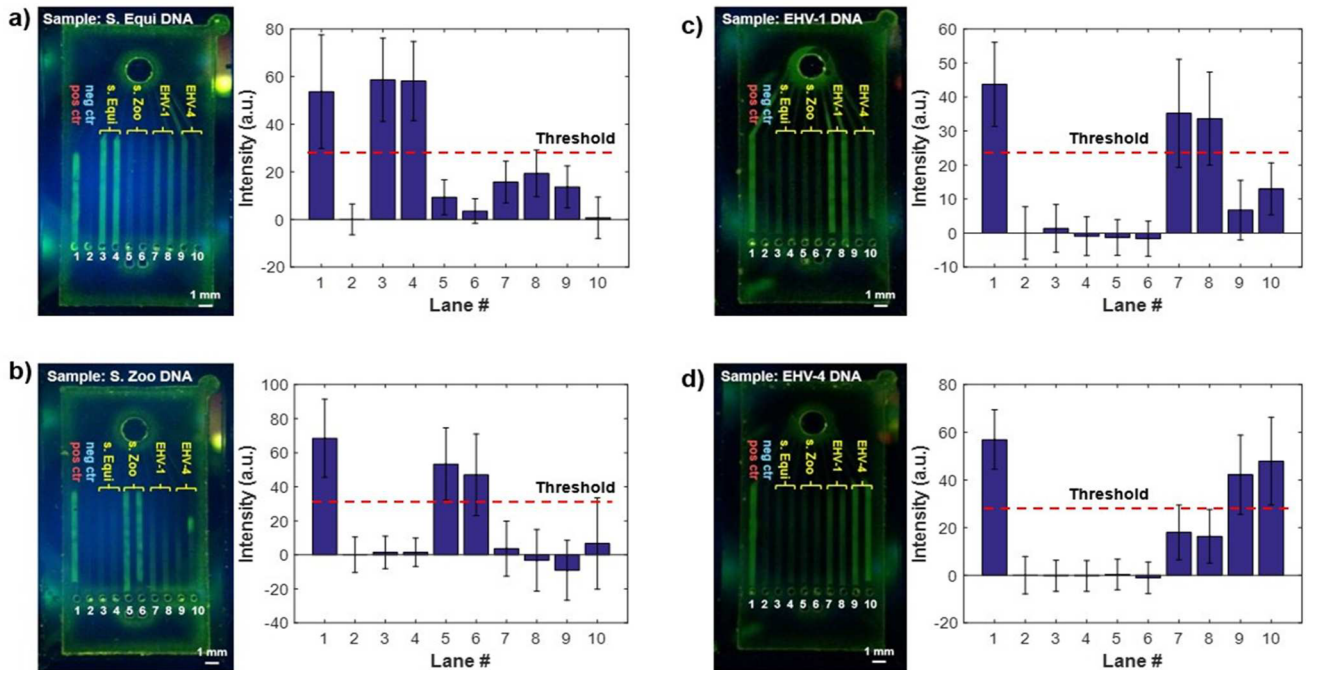

Figure 3. Experiments demonstrating one-at-a-time detection of target DNA sequences. Smartphone-captured images and intensities from each of the ten lanes on the fluorescence images are shown for (a) S. Equi detection, (b) S. Zoo detection, (c) EHV-1 detection, and, (d) EHV-4 detection. The LAMP reactions generate fluorescent output only in the positive control channel and the channels prepared with the specific primers. The presence of specific DNA sequences can be identified by the brightness of the lanes on the fluorescence images.

smartphone-based instrument and the software application, detection results can be quickly analyzed and displayed. The specificity of the LAMP reactions was initially validated when performed in the "on-chip" environment, using a laboratory fluorescence microscope to dynamically image up to six lanes (limit of the microscope field of view) as discussed in the Supporting Information and shown in Figure S5 and Video S1. All assays performed on-chip behaved identically to those performed with the same reagents in a PCR tube. Kinetic tracking of the microscope-measured average fluorescence intensity within a lane as a function of time was used to generate the plots shown in Figures S6–S8, in which the concentration of target DNA copies was manipulated between 5×10^2 and 5×10^6 copies/mL. Detection limits for the on-chip LAMP reaction are compared to those obtained for the off-chip LAMP and PCR reactions in Table 1. Note that 5×10^3 copies/mL represents approximately five nucleic acid target molecules within a lane. Note also that all positive tests, regardless of the concentration above the detection limit, yield identical end point fluorescent intensities as the LAMP reaction saturates. Thus, the on-chip system provides limits of detection comparable to those of the conventional laboratory method for PCR and LAMP.

Figure 3 shows the results for the four experiments performed for the detection of each of the four types of pathogen-specific DNA sequences using the smartphone instrument as the sensor at the end point of the assay only. For the experiment shown in Figure 3a, the sample loaded into the chip contains 5×10^7 copies/mL of S. Equi DNA. As seen in the fluorescence image, lanes 3 and 4, where the S. Equi primers are located, become as bright as the positive control (lane 1). To quantitatively analyze the fluorescence image, the average intensities of the 10 lanes, as processed in the method described in the "Image analysis and app development" section in the Supporting Information, are calculated. Here, we designate a threshold value to equal half of the intensity of the positive control, as the value that is used to differentiate a positive test (target DNA present) from a negative test (target DNA not present). As shown in the bar graph in Figure 3a, only the intensities of lanes 3 and 4 are significantly higher than the threshold, while the intensities of all the other lanes are well below the threshold. The variation in the intensities of the negative lanes for lanes 5-10 is due to background fluorescence from unreacted EvaGreen dye molecules. The error bar in the figure represents the standard deviation of the pixel intensities within each lane. Similar experiments were performed with the target DNA sequences from the other three pathogens. In Figure 3b, the concentration of the injected S. Zoo DNAs is 5×10^6 copies/mL. In accord with the prediction, after the on-chip reactions, lanes 5 and 6 stand out clearly. Panels c and d of Figure 3 show the results for detection of target DNA sequences from viral pathogens EHV-1 and EHV-4. Both experiments show clear evidence that the presence of target DNA can be specifically identified without inducing amplification in nontarget lanes. The concentrations for the injected EHV-1 and EHV-4 DNAs were 5×10^5 and 2×10^6 copies/mL, respectively. Application of the thresholding criterion enables the system to differentiate positive from negative results in each lane.

Detection of Co-infection with Two Target DNA Sequences Present in the Same Sample. Due to the increased risks brought by co-infections and the fact that the same clinical symptom can be caused by infections from several agents, a POC test which can simultaneously detect multiple pathogen-specific target sequences from a single specimen is highly desirable.[37] To further demonstrate the mobile genetic detection platform, we sought to introduce two target DNA sequences into the same test sample and to detect a positive

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response from only the corresponding lanes in the microfluidic chip. The sample prepared for this experiment contains 8×10^4 copies/mL EHV-1 DNAs and 3.2×10^5 copies/mL EHV-4 DNAs, while the microfluidic chip is prepared as described in the Supporting Information. Results from this test are shown in Figure 4. As shown by the fluorescence image captured by the

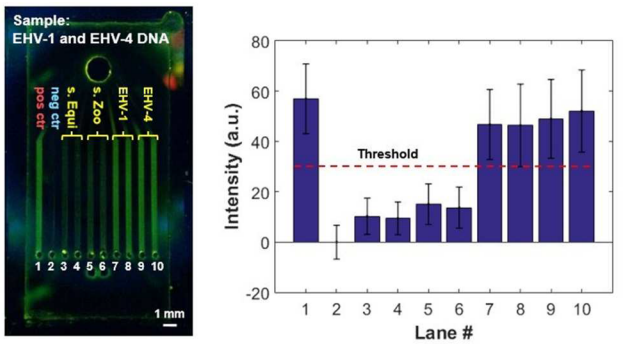

Figure 4. Demonstration of co-detection of multiple pathogen DNA sequences. The sample contains both the EHV-1 and EHV-4 DNA targets. Lane 1 (the positive control), lanes 7 and 8 (the lanes for EHV-1), and lanes 9 and 10 (the lanes for EHV-4) become bright after the on-chip reactions.

smartphone, the high-intensity lanes can be clearly identified as lane 1, the positive control; lanes 7 and 8, which indicate the presence of EHV-1 DNA; as well as lanes 9 and 10, which indicate the presence of EHV-4 DNA. The lanes corresponding to the positive targets are significantly brighter than the negative control and the nontarget lanes, and the thresholding method is able to differentiate between positive and negative results.

DISCUSSION

As described in the introduction, an important application for the technology platform presented in this work is for mobile diagnostics of pathogen-induced equine disease. Respiratory disease is common in horses and difficult to diagnose using the current methods available to practicing veterinarians. Currently there is little to no monitoring of the health of horses even though they are the most valuable of livestock. Some respiratory conditions such as inflammatory airway disease (IAD) and interstitial lung disease are poorly understood although animals are predisposed to these conditions by viral and bacterial infections,[38,39] which occur worldwide.[40] Miniaturized and portable DNA analysis methods have emerged as promising tools for veterinary infectious diseases. State-of-the-art miniaturized systems that perform DNA amplification reactions can be classified as small thermocyclers with integrated photodiodes,[41] microfluidic systems with customized optics for automated and multiplexed experiments,[42,43] and systems based on electrochemical principles that achieve miniaturization by removing optical elements.[44,45] Small thermocyclers such as "Palm PCR"[41] let the user perform the reaction in a pocket-size device, but all the sample preparation is still performed by the user and there are no multiplexing capabilities. The microfluidic "FilmArray"[42] system is highly automated and considerably reduces sample preparation and handling. However, the instrument size and complexity results in an expensive benchtop system that is unsuitable for portable applications. Electrochemical-based systems such as the one demonstrated by DNA Electronics[44] monitors variables such as pH change or secondary redox reactions to assess DNA amplification without optical elements. However, these alternatives require the compartmentalization of reactions since the monitoring variables are not specific to the target, complicating assay multiplexing.

We envision our system to be compatible with existing DNA/RNA extraction kits that are commercially available for PCR without the need for additional laboratory capabilities. We also envision using the system in the context of a mobile equine laboratory that would have a centrifuge and hot plate available for sample preprocessing. Therefore, centrifugation and heat lysis were used to rapidly extract tested material from viral/bacterial culture media for the results reported here. Because many inhibitors to the amplification−reaction may be present in a sample (such as a nasal swab or whole blood), extraction kits with 15 min protocols have been extensively engineered to improve the detection accuracy and have been demonstrated as an effective tool for extracting pathogenic DNAs from horse saliva samples with good sensitivity, and thus they represent a viable option for point-of-use testing.[46] In this work, heat lysis is chosen as the method of DNA extraction due to the simple protocol, short processing time, and low cost. LAMP assays using unfiltered sample material can be performed without loss of specificity.[47,48] Due to the robustness of Bst Polymerase against contaminants, LAMP has been already been used for the direct amplification of analytes from whole blood[23,49] and saliva[44] with minimal sample processing at the point-of-care. We hope to integrate these strategies when we are handling clinical samples in subsequent studies.

We found that selecting the positive/negative threshold as half the difference of the positive and negative controls provided zero false positive and false negative determinations of the presence of the target pathogen within a lane. Note that all target DNA concentrations above the limits of detection yield approximately the same end point fluorescence intensity after a 30 min LAMP reaction, and thus the system is intended only to make determinations about the presence or absence of a pathogen, rather than to estimate its concentration.

A very useful capability for a mobile veterinary laboratory and for human POC diagnosis is to simultaneously test for the presence of more than one pathogen with a single test protocol, which lowers cost, saves time/effort, and allows for a panel of pathogens, which may cause similar symptoms, to be identified. Multiplex LAMP or PCR assays using multiple primer sets in the same reaction can decrease the consumption of sample and reagent but are limited in detection sensitivity caused by uneven amplification efficiencies of the different primer sets.[37] The approach developed in this project splits the initial sample into multiple parallel flow channels for multiplexed LAMP-based detection. Only 30 min is needed to complete the on-chip amplification reactions, which can be performed independently from the phone using a simple hot plate or oven that can process many "cards" at a time. Thus, the smartphone is only occupied during the <5 s reading phase of the assay, leaving its other functions available to the user.

The incorporation of a smartphone in the detection platform not only reduces the cost of the detection system but also enables streamlined integration of the sensing function with a mobile network for infectious disease epidemiology. Therefore, we envision that a user is interested not only in the results of tests that they conduct themselves but also in the results of a network of similar users who perform testing on geographically distributed patients. The detection results can be collected by

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the smartphone app and transmitted through the mobile network for the reporting of the pathogens or further analysis at centralized laboratories, interpretation by remote physicians, or by physicians interested in epidemiological trends. Due to the portability of the smartphone-based system, the detection can be quickly deployed and performed in a wide range of regions upon the outbreaks of infectious diseases, especially in resource-poor environments. On the other hand, the same regions or countries, where the public health system has broken down, often face the greatest risk of the emergence of new and once-controlled infectious diseases. A low-cost, portable, and smartphone-integrated system provides a promising solution to address the challenges of infectious disease diagnostics in resource-limited settings.

We have established a database system to collect the readings from the smartphone app and to gather information regarding the users and patients. Test information in the form of positive/negative determinations and intensity percentile for all assays, as well as all user/patient information, can be wirelessly transmitted to the data management system. MongoDB, an open-source document-oriented database, is selected as the platform due to its high performance, high availability, and automatic scaling. The data are recorded using a SOAP (subjective, objective, assessment, and plan) format approach. Subjective data include a patient's basic information such as name, age, sex and location, while objective data include clinical signs such as heart rate, rectal temperature, and body condition. "Assessment" lists the problems for the patient, current diagnosis, and staging of the disease. "Plan" indicates the actions to be taken. The SOAP note format can provide a problem-solving structure for the users of the database. By creating such a data visualization tool, the users are able to select, prioritize, and view the results of tests that can be screened by tags that include location, disease, and patient type. The system can support direct messaging communication to other users and selection of alerts for specific diseases when positive readings occur. The establishment of a data management system for the mobile detection instrument can provide physicians, veterinarians, and public health authorities with the ability to monitor outbreaks, screen patients, and conduct follow-up tests.

CONCLUSION

We demonstrated a compact, rapid, multiplexed, and inexpensive system for smartphone-based detection and identification of disease-specific nucleic acid sequences within a single-droplet test sample. The system utilizes a microfluidic approach for performing LAMP-based isothermal amplification of a multiplexed array of 1−10 pathogen-specific nucleic acid sequences, and uses a hand-held cradle that interfaces with the rear-facing camera of a conventional smartphone to capture the fluorescence images. The captured images are analyzed by a smartphone app and shared to a cloud-based database for rapid reporting of the detection results. Four LAMP assays have been developed for detection of the specific genes of four major pathogens that cause equine respiratory infectious diseases, including *Streptococcus equi*, *Streptococcus zooepidemicus*, and *Equine herpesvirus* types 1 and 4. As compared with the assays performed on a conventional laboratory thermocycler, the detection sensitivity is not compromised using the microfluidic approach and the smartphone-based instrument. Importantly, the system is capable of detecting multiple nucleic acid targets at the same time and, thus, is capable of identifying co-infections of multiple pathogen strains. By generating a positive/negative determination of the presence of specific pathogens with integrated experimental controls and replicates, the mobile system can assist physicians in rapid point-of-care decision-making for treatment and quarantine response that is currently not possible with tests performed at central laboratory facilities. We believe this approach provides a mobile, simple, and inexpensive capability for clinicians to perform infectious disease diagnostics, and it represents a significant stride toward a practical solution to infectious disease diagnostics in resource-limited settings.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.7b02478.

Real-time measurement of on-ship reactions for S. Zoo target DNA detection (AVI)

Silicon microfluidic channel photolithography and etching; chip preparation and sealing; LAMP assay development; fluorescence microscopy; image analysis and app development; off-chip characterization of the LAMP assay; on-chip characterization of the LAMP assay; off-chip verification of assay specificity; real-time measurement of the on-chip reactions; and sequences of primers used in the LAMP assays (PDF)

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) CDC. *Popul. Dev. Rev.* **1999**, *25*, 635−640.

(2) Radostits, O. M., Gay, C. C., Hinchcliff, K. W., Constable, P. D., Eds. *Veterinary Medicine E-Book: A textbook of the diseases of cattle, horses, sheep, pigs and goats*, 10th ed.; Elsevier Health Sciences, 2006.

(3) Sachs, J.; Malaney, P. *Nature* **2002**, *415*, 680−685.

(4) Neumann, E. J.; Kliebenstein, J. B.; Johnson, C. D.; Mabry, J. W.; Bush, E. J.; Seitzinger, A. H.; Green, A. L.; Zimmerman, J. J. *J. Am. Vet. Med. Assoc.* **2005**, *227*, 385−392.

(5) Ferguson, N. M.; Cummings, D. A.; Fraser, C.; Cajka, J. C.; Cooley, P. C.; Burke, D. S. *Nature* **2006**, *442*, 448.

(6) Lee, S. H.; Kim, S. W.; Kang, J. Y.; Ahn, C. H. *Lab Chip* **2008**, *8*, 2121−2127.

(7) Wang, J.; Chen, Z. Y.; Corstjens, P. L. A. M.; Mauk, M. G.; Bau, H. H. *Lab Chip* **2006**, *6*, 46−53.

DOI: 10.1021/acs.analchem.7b02478
Anal. Chem. XXXX, XXX, XXX−XXX

(8) Zhang, C. S.; Xing, D. *Nucleic Acids Res.* **2007**, *35*, 4223−4237.
(9) Ahmad, F.; Hashsham, S. A. *Anal. Chim. Acta* **2012**, *733*, 1−15.
(10) Zhang, Y. H.; Ozdemir, P. *Anal. Chim. Acta* **2009**, *638*, 115−125.
(11) Compton, J. *Nature* **1991**, *350*, 91−92.
(12) Walker, G. T.; Fraiser, M. S.; Schram, J. L.; Little, M. C.; Nadeau, J. G.; Malinowski, D. P. *Nucleic Acids Res.* **1992**, *20*, 1691−1696.
(13) Piepenburg, O.; Williams, C. H.; Stemple, D. L.; Armes, N. A. *PLoS Biol.* **2006**, *4*, e204.
(14) Stedtfeld, R. D.; Tourlousse, D. M.; Seyrig, G.; Stedtfeld, T. M.; Kronlein, M.; Price, S.; Ahmad, F.; Gulari, E.; Tiedje, J. M.; Hashsham, S. A. *Lab Chip* **2012**, *12*, 1454−1462.
(15) Duarte, C.; Salm, E.; Dorvel, B.; Reddy, B.; Bashir, R. *Biomed. Microdevices* **2013**, *15*, 821−830.
(16) Craw, P.; Balachandran, W. *Lab Chip* **2012**, *12*, 2469−2486.
(17) Fang, X.; Liu, Y.; Kong, J.; Jiang, X. *Anal. Chem.* **2010**, *82*, 3002−3006.
(18) Fang, X.; Chen, H.; Yu, S.; Jiang, X.; Kong, J. *Anal. Chem.* **2011**, *83*, 690−695.
(19) Zhang, Y.; Zhang, L.; Sun, J.; Liu, Y.; Ma, X.; Cui, S.; Ma, L.; Xi, J. J.; Jiang, X. *Anal. Chem.* **2014**, *86*, 7057−7062.
(20) Parida, M.; Sannarangaiah, S.; Dash, P. K.; Rao, P. V. L; Morita, K. *Rev. Med. Virol.* **2008**, *18*, 407−421.
(21) Chen, W. L.; Long, K. D.; Kurniawan, J.; Hung, M.; Yu, H. J.; Harley, B. A.; Cunningham, B. T. *Adv. Opt. Mater.* **2015**, *3*, 1623−1632.
(22) Chen, W. L.; Long, K. D.; Yu, H. J.; Tan, Y. F.; Choi, J. S.; Harley, B. A.; Cunningham, B. T. *Analyst* **2014**, *139*, 5954−5963.
(23) Damhorst, G. L.; Duarte-Guevara, C.; Chen, W.; Ghonge, T.; Cunningham, B. T.; Bashir, R. *Engineering* **2015**, *1*, 324−335.
(24) Peterson, R. D.; Chen, W. L.; Cunningham, B. T.; Andrade, J. E. *Biosens. Bioelectron.* **2015**, *74*, 815−822.
(25) *Global Analysis of the Smartphones Market: Who's Winning the Necessary Regional Battles*; Frost & Sullivan, 2013.
(26) Comtois, G.; Salisbury, J. I.; Sun, Y. *2012 38th Annual Northeast Bioengineering Conference (NEBEC)*; IEEE, 2012; pp 69−70, DOI: 10.1109/NEBC.2012.6206966.
(27) Huang, C.-C.; Lee, P.-Y.; Chen, P.-Y.; Liu, T.-Y. *IEEE Trans. Ultrason., Ferroelect., Freq. Contr.* **2012**, *59* (1), 182−188.
(28) Breslauer, D. N.; Maamari, R. N.; Switz, N. A.; Lam, W. A.; Fletcher, D. A. *PLoS One* **2009**, *4*, e6320.
(29) Wei, Q. S.; Qi, H. F.; Luo, W.; Tseng, D.; Ki, S. J.; Wan, Z.; Gorocs, Z.; Bentolila, L. A.; Wu, T. T.; Sun, R.; Ozcan, A. *ACS Nano* **2013**, *7*, 9147−9155.
(30) Gallegos, D.; Long, K. D.; Yu, H. J.; Clark, P. P.; Lin, Y. X.; George, S.; Nath, P.; Cunningham, B. T. *Lab Chip* **2013**, *13*, 2124−2132.
(31) Jiang, J.; Wang, X. H.; Chao, R.; Ren, Y. K.; Hu, C. P.; Xu, Z. D.; Liu, G. L. *Sens. Actuators, B* **2014**, *193*, 653−659.
(32) Yu, H.; Tan, Y.; Cunningham, B. T. *Anal. Chem.* **2014**, *86*, 8805−8813.
(33) Long, K. D.; Yu, H.; Cunningham, B. T. *Biomed. Opt. Express* **2014**, *5*, 3792−3806.
(34) Kwon, L.; Long, K. D.; Wan, Y.; Yu, H.; Cunningham, B. T. *Biotechnol. Adv.* **2016**, *34*, 291−304.
(35) Wan, Y.; Carlson, J. A.; Kesler, B. A.; Peng, W.; Su, P.; Al-Mulla, S. A.; Lim, S. J.; Smith, A. M.; Dallesasse, J. M.; Cunningham, B. T. *Sci. Rep.* **2016**, *6*, 29117.
(36) Yu, H.; Le, H. M.; Kaale, E.; Long, K. D.; Layloff, T.; Lumetta, S. S.; Cunningham, B. T. *J. Pharm. Biomed. Anal.* **2016**, *125*, 85−93.
(37) Park, S.; Zhang, Y.; Lin, S.; Wang, T.-H.; Yang, S. *Biotechnol. Adv.* **2011**, *29*, 830−839.
(38) Rush, B.; Mair, T. *Equine Respiratory Diseases*; Wiley-Blackwell, 2004.
(39) Lavoie, J.-P.; Hinchcliff, K. W. *Blackwell's Five-Minute Veterinary Consult: Equine*; John Wiley & Sons, 2008.
(40) Couetil, L.; Hawkins, J. F. *Respiratory Diseases of the Horse: A Problem-Oriented Approach to Diagnosis and Management*; CRC Press, 2013.
(41) Love, J. M.; Marquis-Nicholson, R.; Love, R. C.; Love, D. R. *Res. J. Biol.* **2012**, *2*, 191−196.
(42) Poritz, M. A.; Blaschke, A. J.; Byington, C. L.; Meyers, L.; Nilsson, K.; Jones, D. E.; Thatcher, S. A.; Robbins, T.; Lingenfelter, B.; Amiott, E.; Herbener, A.; Daly, J.; Dobrowolski, S. F.; Teng, D. H.-F.; Ririe, K. M. *PLoS One* **2011**, *6*, e26047.
(43) Abe, T.; Segawa, Y.; Watanabe, H.; Yotoriyama, T.; Kai, S.; Yasuda, A.; Shimizu, N.; Tojo, N. *Lab Chip* **2011**, *11*, 1166−1167.
(44) Toumazou, C.; Shepherd, L. M.; Reed, S. C.; Chen, G. I.; Patel, A.; Garner, D. M.; Wang, C.-J. A.; Ou, C.-P.; Amin-Desai, K.; Athanasiou, P.; Bai, H; Brizido, I. M. Q.; Caldwell, B.; Coomber-Alford, D.; Georgiou, P.; Jordan, K. S.; Joyce, J. C.; La Mura, M.; Morley, D.; Sathyavruthan, S.; Temelso, S.; Thomas, R. E.; Zhang, L. *Nat. Methods* **2013**, *10*, 641−646.
(45) Patterson, A. S.; Hsieh, K.; Soh, H. T.; Plaxco, K. W. *Trends Biotechnol.* **2013**, *31*, 704−712.
(46) Kinoshita, Y.; Niwa, H.; Katayama, Y. *J. Vet. Med. Sci.* **2014**, *76*, 1271−1275.
(47) Ishiguro, N.; Koseki, N.; Kaiho, M.; Kikuta, H.; Togashi, T.; Watanabe, T.; Ariga, T. *Clin. Lab.* **2015**, *61*, 603−606.
(48) Poon, L. L. M.; Wong, B. W. Y.; Ma, E. H. T.; Chan, K. H.; Chow, L. M. C.; Abeyewickreme, W.; Tangpukdee, N.; Yuen, K. Y.; Guan, Y.; Looareesuwan, S.; Peiris, J. S. M. *Clin. Chem.* **2006**, *52*, 303−306.
(49) Priye, A.; Bird, S. W.; Light, Y. K.; Ball, C. S.; Negrete, O. A.; Meagher, R. J. *Sci. Rep.* **2017**, *7*, 44778.

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