Rapid Veterinary Diagnosis of Bovine Reproductive Infectious Diseases from Semen using Paper-Origami DNA Microfluidics

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ABSTRACT. The health and well-being of cattle is a significant concern for global agricultural output. In dairy production within low and middle income countries (LMICs), there is a significant biosensing challenge in detecting sexually transmitted infection (STI) pathogens during animal husbandry, due in part to difficulties associated with the limited infrastructure for veterinary medicine. Here we demonstrate low-cost, multiplexed and sample-to-answer paper-origami tests for the detection of three bovine infectious reproductive diseases in semen samples, collected at a test site in rural India. Pathogen DNA from one viral pathogen, Bovine Herpes virus-1 (BoHV-1) and two bacteria (*Brucella* and *Leptospira*) was extracted, amplified (using loop-mediated isothermal amplification, LAMP) and detected fluorescently, enabling <1 pg (~ from 115 to 274 copies per reaction) of target genomic DNA to be measured. Data was collected as a fluorescence signal either visually, using a low-cost hand-held torch, or digitally with a mobile-phone camera. Limits of detection and sensitivities of the paper-origami device for the three pathogens were also evaluated using pathogen-inoculated semen samples and were as few as 50 *Leptospira* organisms, 50 CFU *Brucella* and 1 $TCID_{50}$. BoHV-1. Semen samples from elite bulls at a germplasm centre were also tested in double-blind tests, as a demonstrator for a low cost, user-friendly point-of-care sensing platform, for in-the-field resource-limited regions. The sensors showed excellent levels of sensitivity and specificity, and for the first time a demonstrated ability of the application of paper-origami devices for the diagnosis multiple infectious diseases from semen samples.

The transmission of sexually transmitted infection (STI) pathogens amongst dairy animals is a significant concern for global agriculture output[1]. These reproductive diseases show significantly high prevalence in low and middle income countries (LMICs), which often lack the required infrastructure and facilities for veterinary medicine, leading to infected animals either not being detected or, if identified, being diagnosed at a sufficiently late stage that the infection has spread. Insemination is an important route of transmission, particularly when performed artificially, and may result in either acute disease or in long-term reproductive complications. In both cases, adverse outcomes in the pregnancy range from abortion to the infection of the offspring [2,3].

Among infectious pathogens causing abortion and reproductive failure in cattle and buffaloes, the bacterial pathogens *Brucella* and *Leptospira* and viral pathogen bovine herpes virus-1 (BoHV-1) play significant roles. Prevalence of these diseases are particularly high in many tropical and subtropical regions, including India. Established detection methods for these pathogens include isolation of the organisms[4,5] and serological assays[6]. However, these techniques are either time-consuming, requiring well-equipped laboratory and skilled manpower, preventing their deployment in the field or in insemination facilities, or lack sufficient sensitivity[7]. In particular, in India, where this study was carried out, animals are required to be tested at germplasm stations for specific pathogens, which could be transmitted to the female and further to the offspring.

As a consequence, there is now an economic and veterinary need to develop sensitive and low cost platforms for the direct detection of STI pathogens in semen samples, for samples freshly collected at bull stations or in prepared frozen semen straws. Direct direction in semen has the potential to increase reliability, where immunoassays have been shown to generate false negative results[8]. Such a platform may not only have a direct economic benefit but may also provide useful epidemiological information in exploring disease prevalence and routes of transmission in other rural situations[9]. Veterinary testing is not the only important diagnosis, as leptospirosis and brucellosis[10–12] both also have a significant global zoonotic impact, with animal workers, veterinarians, abattoir workers and farmers all at a high risk of infection[10]. Infectious bovine rhinotracheitis (IBR) is also a highly contagious, infectious respiratory

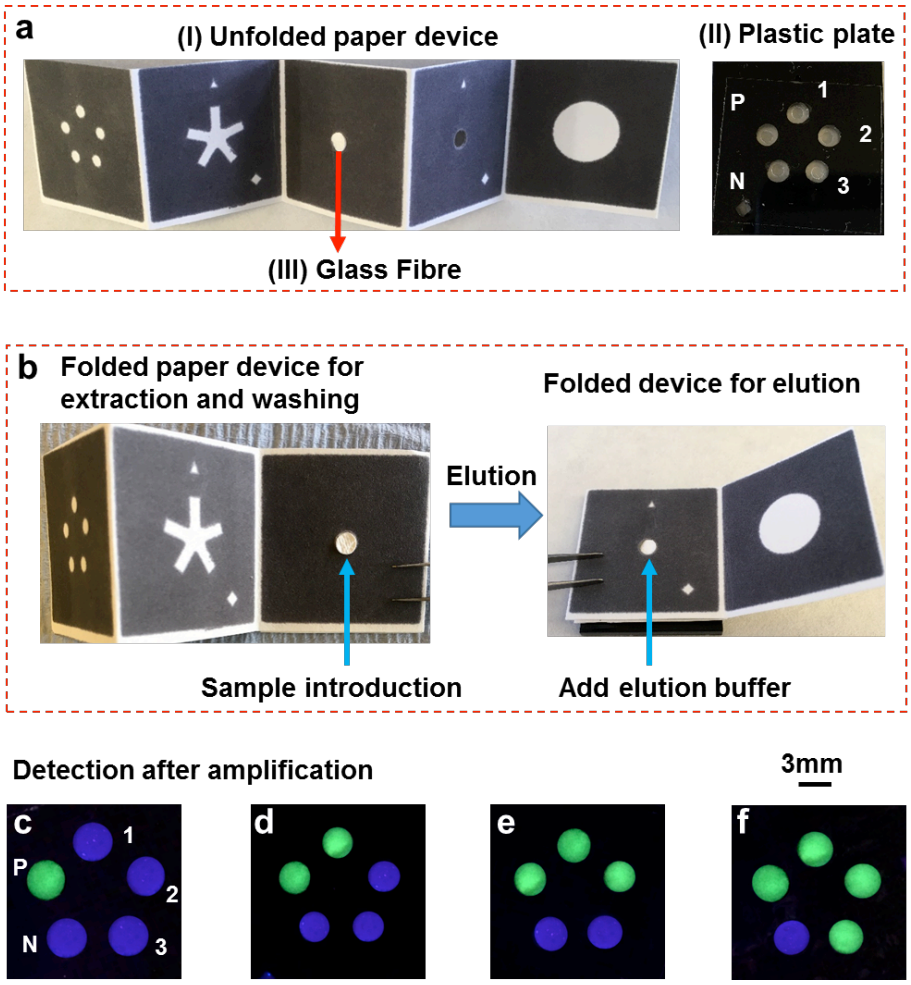

Figure 1 Design of the paper device for the detection of 3 targets (1, 2 and 3) with internal positive (P) and negative control (N). (a) The device contains three components (**I**) a filter paper based fluidic device with wax-printed fluidics channels, (**II**) a plastic plate with 5 LAMP reaction chambers (P, N, 1, 2 and 3 representing 5 reaction chambers in plastic plate, corresponding to the image of P, N, 1, 2 and 3 showing in Figure 1 (c)); and (**III**) one glass fibre circular disk. The unfolded device consists of 5 panels folding onto each other. It is mounted to a plastic plate (**II** in Figure 1(a)) with a cover to avoid evaporation. The design also incorporates alignment marks on two corners (diamond on bottom and triangle in top middle) to assign the results. (b) The sample was introduced into glass fibre and DNA was extracted, followed by washing and eluting to the reaction chamber of plastic plate for LAMP reaction. After amplification, the signal is read out with a UV flashlight (365 nm). (c - f) The green emission occurs in the presence of pyrophosphate under UV excitation. (c) blank sample; the labels denote the different species-specific LAMP reaction, P: internal positive control; N: internal negative control; 1: *Leptospira*; 2: *Brucella*; 3: BoHV-1; (d-f) are representative images taken after the processing of DNA spiked in buffer for single (*Leptospira* - d), duplex (*Leptospira* and *Brucella* - e) and triplex (*Leptospira*, *Brucella* and BoHV-1 - f) target detection (2 ng μl^{-1}), together with the positive (2 ng μl^{-1} of BRCA1 genomic DNA, a sequence from a human gene used as a positive control – see Table S1 for details) and negative controls to check if the test is valid (green color for P and no green for N). The whole process is illustrated in Figure S1.

disease that is caused by BoHV-1 and which can cause conjunctivitis, abortions, encephalitis and generalized systemic infections[14].

Nucleic acid based tests (NATs) are now an important tool for healthcare providers and veterinary scientists to detect infections rapidly, in laboratories but also at the point-of-care, providing excellent sensitivity and specificity. The gold standard technique remains the polymerase chain reaction (PCR)[13,15], which is both faster and more specific than culture techniques (taking only a few hours compared to days or weeks and able, for example to clearly identify resistant strains). However, PCR still requires a costly thermocycler and skilled personnel to establish the assay and analyse the data, thus, limiting its application in LMICs. As an alternative that does not require complex temperature cycling, decreasing the requirements for well-equipped facilities, loop-mediated isothermal amplification, LAMP, has emerged as a powerful isothermal means of performing NATs yielding high specificity and sensitivity[16,17].

LAMP has recently been used to rapidly detect *Brucella*[18], *Leptospira*[19] and BoHV-1[3] as single assays performed under laboratory conditions. LAMP has also been carried out within microfluidic chips[20] and capillary platforms[21,22] demonstrating the potential of the technique for point-of-care diagnosis. In this latter respect, we have recently implemented LAMP assays on paper origami microfluidic devices[13], which use capillary flows to move liquids around 2D and 3D microchannel networks, without the need for external pumps or power supplies – for the detection of two *Plasmodium* sp. from finger-prick volume of whole blood from 80 patients (as a triplex,

including *Plasmodium* pan). Folding of the paper enabled us to integrate DNA extraction, amplification and visual detection. Different implementations of paper micro-devices, ranging from chemical analysis to multiplexed immunoassays have, together with our work[13] shown the potential in bringing complex assay workflows to low-resource settings.[23,24] However, existing devices are complex both in manufacture and is use, inappropriate to field studies in a low resource environment,[25–27] and while origami has been used in bioprocessing before[28], it has only previously been used for nucleic acid technologies in our previously published laboratory-based study[13]. Origami has not previously been used in field studies in a low resource environment.

Here we now demonstrate a new design of the paper-origami device (Figure 1) for veterinary applications, with multiplexing through 5 channels, to enable the detection of three target pathogens/virus (*Brucella*, *Leptospira* and BoHV-1) – see Figure S1 for the complete process. To increase the confidence in the results, essential when being used by unskilled individuals, we now also include both negative and positive internal controls (the latter being a sequence of human genomic DNA, BRCA1), to minimise reporting of the false positive and false negative results[16,17]. The incorporation of the two controls also enables the normalisation of the output signal intensity across devices, thus enabling real-time and quantitative analysis.

The performance was tested in fresh and frozen semen samples collected at the germplasm centre, Indian Veterinary Research Institute (IVRI) in India. This novel paper device achieved excellent analytical performance in real semen samples with a limit of detection of 50 *Leptospira* organisms, 50 CFU *Brucella abortus* (*bcsp 31*) and 1 TCID50 for BoHV-1 per reaction, results which are comparable or better than gold-standard tests. To our knowledge this is the first demonstration of a paper microfluidic diagnostic device using semen, and also its first demonstration in a low resource, rural setting as a demonstration of early diagnosis of bovine STIs in the field in low-income areas.

EXPERIMENTAL SECTION

Materials. Gsp SSD DNA polymerase (large fragment), $MgSO_4$ and betaine were from OptiGene Ltd. (UK). EvaGreen™ was from Biotium (UK). All the primers were synthesized by Eurofins (UK), detailed in Table S1 in ESI. SYBR® Gold Nucleic Acid Gel Stain was from Life Technologies (UK). Deoxy-ribonucleoside triphosphates (dNTPs) and other reagents were from Sigma-Aldrich (UK).

Loop mediated isothermal amplification (LAMP) assay. Primers were tested for hybrids and hairpin structures using the Integrated DNA Technologies design tools (http://eu.idtdna.com/pages/scitools). The optimization of the LAMP assay was performed on a real-time PCR machine (Applied Biosystems, Thermal Fisher Scientific, UK). The LAMP amplicon was analysed on 1% (w/v) agarose gel in TAE 1x buffer (pH 8) and the image was recorded by a gel reader (See Figure S2 in ESI).

The concentration of primers sets for three pathogen target were detailed in Table S1 (ESI), and an internal positive control (*BRAC1* gene) was introduced with human genomic DNA as the template. The mix also contained 0.4 mM of each dNTPs (Sigma), 4.0 mM of $MgSO_4$, 50 mM Tris-HCl (pH 8.1), 30 mM KCl, 30 mM $(NH_4)_2SO_4$, 0.1 % Triton X-100, 1 M of betaine, 25 µM calcein (Sigma), and 500 µM $MnCl_2$. The internal negative control was performed with the same composition with positive control, but using a ddH_2O as the template. The multiplex LAMP reactions were performed at 63 °C for 45 minutes on a hotplate. The results were read out with a handheld-UV lamp (wavelength 365 nm). The calcein in the reaction mixture initially combines with Mn^{2+} (quenched state). When the amplification reaction proceeds, Mn^{2+} complexes with the $P_2O_7^{4-}$ generated, which results in the emission of fluorescence, strengthened by the presence of Mg^{2+} in the reaction mixture[29,30]. The green fluorescence signal intensity was captured using a mobile phone camera (iPhone SE 64 GB) and extracted with the software ImageJ[31]. The average intensity for the whole area was extracted by creating a region of interest - ROI, normalised with respect to the positive and negative controls where the positive control was assigned 1 as an arbitrary unit and the negative control, 0.

Paper-origami devices. The paper-origami devices were produced by hot wax printing 5 channels into a paper microfluidic platform (including one for the positive control, one for the negative control and 3 for the pathogenic targets). Each device contained three components (Figure 1), namely: a filter paper based[13] fluidic device where the fluid was constrained by printing hydrophobic wax [Xerox, cat# 108R00935] to define the channels [printed with Xerox ColorQube 8570]; a single sided adhesive acetate film [MicroAmp® Optical Adhesive Film, Thermo Scientific] sealed plastic plate with 5 paper spots (cut to a diameter of 4 mm), which form chambers for the LAMP reactions; and one glass fibre circular disk, 4 mm in diameter (GFF, Whatman) for absorbing nucleic acids from the sample in the presence of high concentration of the chaotropic agent, guanidine thiocyanate (GuSCN)

In order to assemble the device, the filter paper was first printed with black wax using an office printer (Xerox Color Qube 8570)[28,34], heated at 120 °C for 1 min on a hot plate to melt the printed wax, allowing for the diffusion through the paper to form the same hydrophobic pattern of channels and vias throughout the thickness of the paper. The glass fibre disk was attached to the paper device by simply forcing it through a 3 mm hole punched into the printed panel (Figure 1a-b). The footprint of the device is 3 cm x 3 cm for each panel (3x 15 cm unfolded). The paper channel is 6 mm x 3 mm.

DNA extraction from semen. 20 µl of semen was mixed in a tube with 40 µl of lysis buffer (1.20 g ml^{-1} of GuSCN, 0.1 M Tris hydrochloride, 0.04 M EDTA, adjusted with NaOH to pH 8.0, 26 mg ml^{-1} Triton X-100[29]), before heating at 95 °C for 5 minutes. The process flow for testing is adapted from our earlier report[13] and shown in supplementary Movie M1. Briefly, 20 µl of the resulting solution was then dispensed onto the paper device, where it permeated the glass fibre and was absorbed by capillary action into the surrounding blotting paper (larger disk in the paper device). The DNA was drawn in this flow and captured by the glass fibre. Subsequently, 100 µl of washing buffer (30mM NaOAc in 70% v/v ethanol) was used to rinse cell residues away. The paper is then folded for elution. As elution buffer (30 µl, 10 mM TE buffer, pH 8.0) is added to the glass fibre, it releases DNA from the glass fibre and contacts the fluidic divider (star-shaped channels), distributing the DNA into the LAMP reaction chambers, where the LAMP reaction mix is added. The paper device is then removed and the chambers sealed with acetate films (MicroAmp® Optical Adhesive Film, Thermo Scientific), preventing liquid evaporation during the amplification.

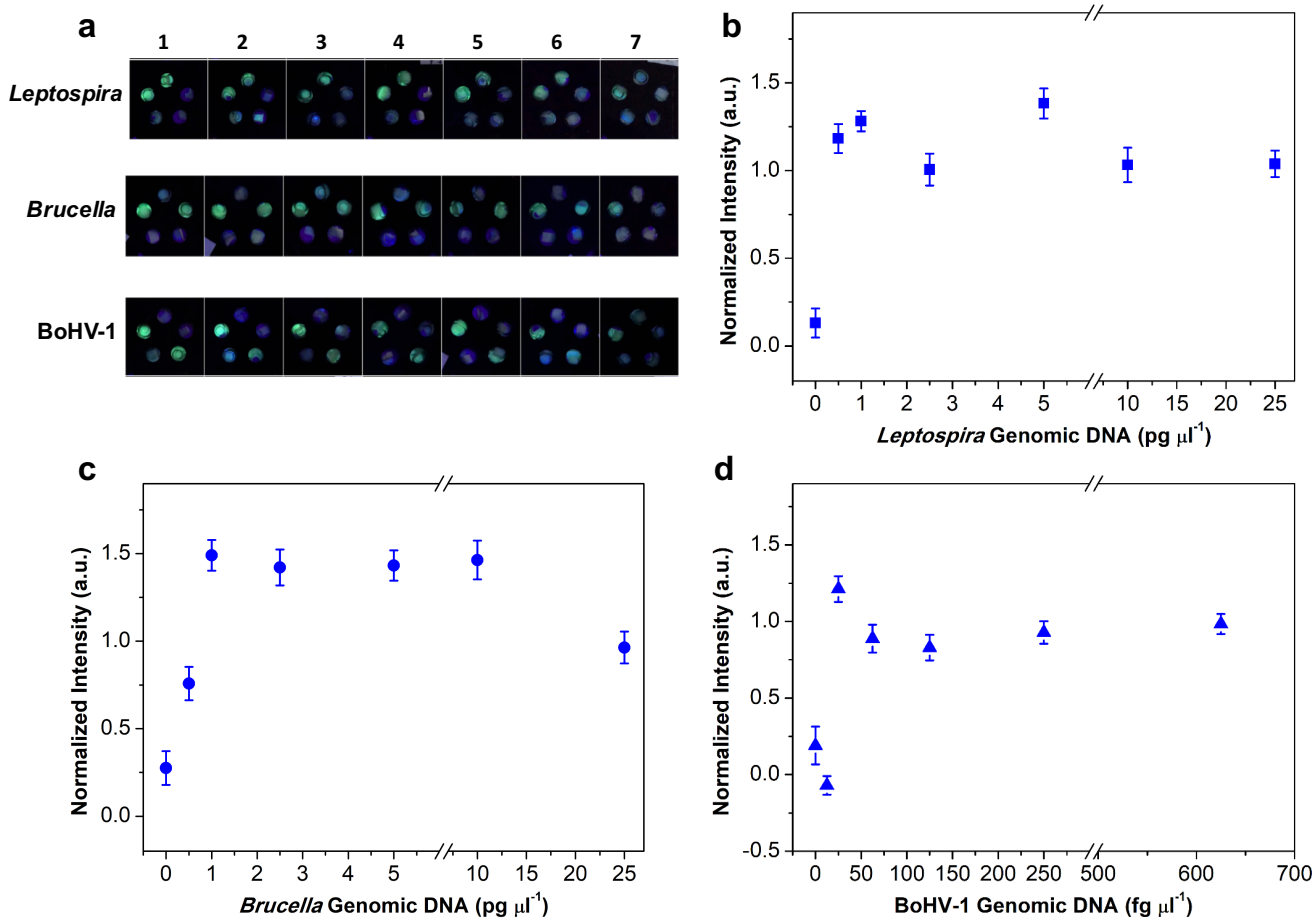

Figure 2. (a) Analytical sensitivity of the paper-origami device for the multiplexed detection of target genomic DNA for the 3 organisms spiked into semen samples. The images are taken after 45 min incubation on the paper device for different concentrations of target DNA spiked in semen (*Leptospira* and *Brucella*: 1. 25 pg μl^{-1}, 2. 10 pg μl^{-1}, 3. 5 pg μl^{-1}, 4. 2.5 pg μl^{-1}, 5. 1 pg μl^{-1}, 6. 0.5 pg μl^{-1}, 7. 0 pg μl^{-1}, BoHV-1: 1. 625 fg μl^{-1}, 2. 250 fg μl^{-1}, 3. 125 fg μl^{-1}, 4. 62.5 fg μl^{-1}, 5. 25 fg μl^{-1}, 6. 12.5 fg μl^{-1}, 7. 0 fg μl^{-1}). The results show a limit of detection of 0.5 pg μl^{-1} for *Leptospira* and *Brucella*, and of 25 fg μl^{-1} for BoHV-1. These values were confirmed by analyzing the intensity of the color signal (b-d), normalized to the controls (we subtracted the intensity of the negative control and then divided by the intensity of the internal positive control), such that a value around 1 indicated a positive test, while values around 0 indicated a negative spot. Error bars are standard deviations of three measurements. The LOD was determined to be 2 σ above background with a confidence interval 95%.

Characterization of the paper device. DNA recovery was tested with a quantitative Taq-man PCR protocol[35] (detailed in ESI). Briefly, 5 different concentrations of double-stranded DNA were spiked into semen samples and extracted with the paper device for q-PCR measurement. The extraction and elution procedures were kept strictly the same as that of the LAMP assay, except for the step of transferring the DNA in the PCR assay, which was done manually. Independent triplicates were performed for the evaluation of DNA recovery, showing a recovery above 60 %.

Pathogens Detection in semen samples. The genomic DNA from the *Brucella abortus*, *Leptospira interrogans* serovar pomona and *E. coli* was extracted using Nucleopore DNA isolation Mini Kit (Genetix, India) and the genomic DNA from Bovine herpesvirus 1 (BoHV-1) was isolated with GeneJET viral DNA & RNA purification kit (Thermo Scientific, Lithuania). DNA concentration was determined with a Nanodrop (Thermo Fisher Scientific, US). Different concentrations of each genomic DNA were spiked into semen samples to characterise the analytical sensitivity of paper-origami devices. *Brucella abortus* S19 strain was obtained from division of biological products, Indian Veterinary Research Institute (IVRI). Single colonies of *Brucella abortus* were grown in tryptose phosphate broth (Difco BD, USA) at 37°C for 72 hours. *Leptospira interrogans* serovar pomona was obtained from *Leptospira* laboratory, Division of Bacteriology, IVRI, India. The culture was grown in liquid EMJH medium (Difco BD, USA) at 28 °C for 4 days. *E.coli* strain DH5α was grown on Luria-Bertani medium (Difco BD, USA) at 37 °C in a shaker incubator for 18 hours.

The extracted genomic DNA was tested with PCR (protocol in ESI) to confirm culturing of each organisms (Figure S2a). For propagation of Bovine herpesvirus 1 (BoHV-1) Indian isolate 216II, Madin-Darby bovine kidney (MDBK) cell line was used.

The organisms (or their genetic material) were spiked into healthy semen samples, collected from 12 elite bulls maintained at the germplasm centre at IVRI, India. Initial screening, prior to spiking, showed that all the samples were negative for *Brucella*, *Leptospira* and BoHV-1 by PCR.

RESULTS AND DISCUSSION

Paper-origami device for LAMP assay of pathogen. The device was fabricated by wax printing[23,36] according to our previous report[13]. Briefly, we exploited the unique mechanical properties of the manual "creasing" of paper in a folding procedure to enable the first steps of the assay, involving cell lysis and DNA extraction, to yield purified DNA on the glass fibre cylinder attached onto the paper panel. The cell lysis step was performed prior to introducing the sample in the device, due to fact the semen is too viscous to move through the paper without dilution. This was followed by transferring DNA from the extraction panel to the amplification panel by "flipping" the paper-fold for elution. The species-specific LAMP reagents of master mix were deposited using micro pipette onto the five independent locations on the paper (labelled in Figure 1). The assembly of the paper device and the operation is illustrated with a video in ESI. Additionally, the size of the filter paper spots was optimised to 4 mm in our device for a maximum DNA recovery above 60 % (Figure S3).

The three reproductive pathogens involved in bovine infectious disease were chosen for the targets[10,14]. Three sets of primers (see Table S1 in ESI) for LAMP were designed for LAMP assay. As shown in Figure S4, the real-time LAMP assay successfully detected concentrations of *Brucella* genomic DNA as low as 0.2 pg μl^{-1} (115 copies per reaction) with a linear range spanning four orders of magnitude, up to 2 ng μl^{-1} (R^2=0.99). The sensitivity for *Leptospira* and BoHV-1 (Figure S4) were 0.4 pg μl^{-1} (170 copies per reaction) and 20 fg μl^{-1} (273 copies per reaction), respectively.

Evaluation of paper-origami device for detection of pathogen DNA in semen samples. To evaluate the feasibility of using the paper-origami device with real samples in India, genomic DNA was extracted from the three pathogens in culture at the IVRI and spiked into bovine semen. Figure 2 shows limits of detection (LOD) at the same level with those obtained in buffer in a real-time configuration on a bench-top machine (Figure S4). The LOD is defined as the concentration of target that can be reliably detected by the device as a positive signal.

To evaluate analytical sensitivity in real samples in the field, different concentrations of cultured organisms were spiked into healthy semen samples collected from a germplasm centre managed by IVRI in India. Fifty colony forming units (CFU) of *Brucella*, Figure 3 (a) (quantified data normalized in Figure 3 (b), (c) and (d)), were detected which is a 4-fold improvement in sensitivity compared with assays using the visual detection of the colour change from hydroxynaphthol blue (HNB) for LAMP assay[18]. The device also enabled the detection of *Leptospira* as low as 50 organisms, a performance which compares favourably with published results[38]. The LOD for BoHV-1 is evaluated to be 1 $TCID_{50}$. A previous study using a visual dye hydroxynaphthol blue (HNB) for a tube-based LAMP assay, demonstrated a LOD for BoHV-1 of 0.2 $TCID_{50}$ on par with our paper device[3].

Screening of semen samples. Typically, the diagnosis of bovine infectious disease is performed using laboratory based methods such as PCR and ELISA on blood samples.[39,40] However, such pathogens usually appear in the blood at a late stage of the infection, making treatment difficult and potentially uneconomical (e.g. more expensive than culling or not treating, especially in LMICs, which lack well-resourced facilities for animal care). Infected or infectious animals are often separated (where possible) or killed in order to reduce the spread of the infection, which leads to additional economic burden.

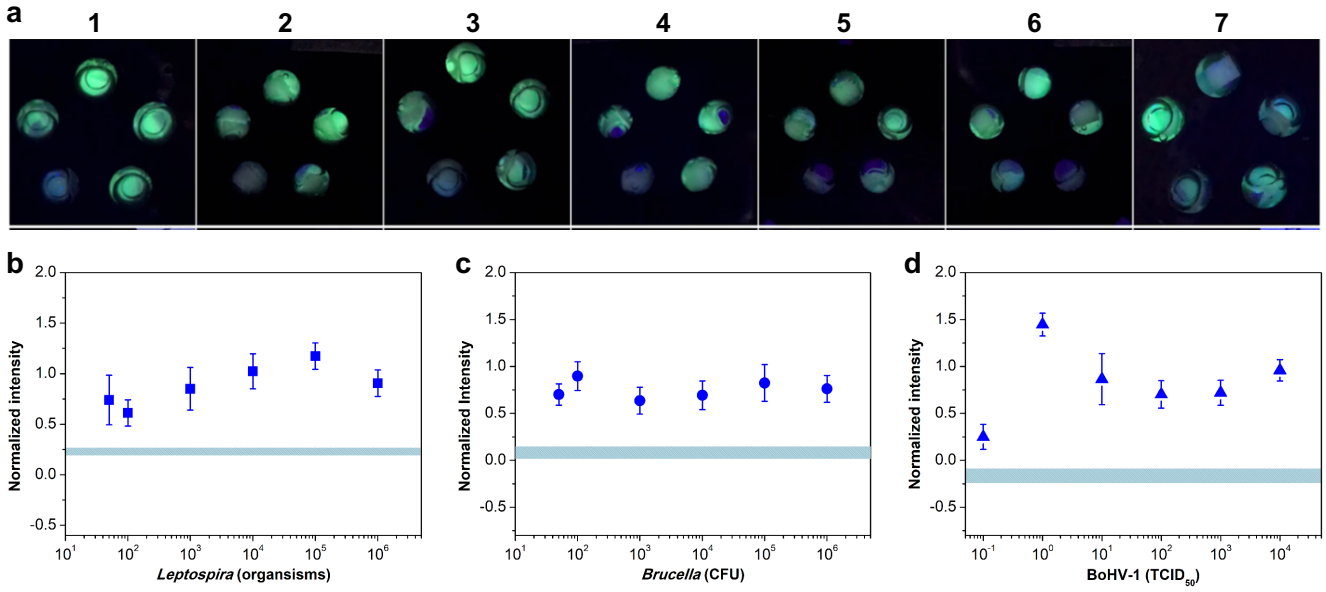

Figure 3. Analytical sensitivity of the paper-origami device for the detection of three organisms spiked into semen samples, evaluated in India. (a) Images of the paper device for the detection of decreasing concentrations of the 3 organisms spiked in semen (from 1-7) for *Leptospira* (top centre spot) 10^6, 10^5, 10^4, 10^3, 10^2, 50 and 0 organisms, and *Brucella* (right spot) at 10^6 CFU, 10^5 CFU, 10^4 CFU, 10^3 CFU, 10^2 CFU, 50 CFU and 0 CFU; and BoHV-1 (bottom right spot) at 10^3 $TCID_{50}$, 10^3 $TCID_{50}$, 10^2 $TCID_{50}$, 10 $TCID_{50}$, 1 $TCID_{50}$, 10^{-1} $TCID_{50}$, 0 $TCID_{50}$, respectively. The green fluorescence signal intensities for *Leptospira* (b), *Brucella* (c) and BoHV-1 (d) were captured using a mobile phone camera (iPhone SE 64 GB) and extracted with the software ImageJ (the average intensity for the whole area was extracted by creating a region of interest - ROI, normalised with respect to the positive and negative controls where the positive control was assigned 1 as an arbitrary unit and the negative control, 0). The LOD was determined with 2σ with a confidence interval 95%. The rectangle in (b), (c) and (e) are signals plus standard deviation assigned to blank (0 organism/CFU/TCID50 in spiked samples) in each figure, respectively.

Paper-origami LAMP devices offer an alternative for a rapid, early and low cost diagnosis is an easy-to-use format, requiring only simple paper folding manipulations.

To illustrate this, semen samples from a cohort of 12 healthy bulls were spiked with live organisms in a small double blind validation study (50 organisms of *Leptospira* per reaction and 50 CFU per reaction *Brucella*, 1 $TCID_{50}$ BoHV-1 as well as 10^3 CFU *E.* coli as a negative control). The captured images were quantified and normalized (Figure S5). All samples were correctly identified by the test and confirmed by electrophoresis image (Figure S2 b), showing that the paper-origami devices hold a great potential for the detection of infections from clinical samples. Results are read with a simple, battery-powered handheld UV lamp, providing a potential solution for testing in the field in resource-limited settings. The assay can also be used with a mobile phone camera, if more precise or sensitive detection is required, for example in therapy monitoring[9,41]. An example of the collection of data in this manner is shown in Figure S5.

CONCLUSION

A new, highly multiplexed paper test for the species-specific detection of pathogens is described providing, for the first time, sample-to-answer STI detection from semen on a paper device. The results show excellent sensitivity and specificity, equivalent or better than the clinical performance published in the literature (although presented as a multiplexed assay for the detection of three pathogens in a single assay). The platform has potential for field testing for the point-of-care diagnosis in human and veterinary subjects and will make it possible for early diagnosis of infectious disease.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.
Supplementary Information (Figures S1-5, Table S1 and associated descriptions) (PDF)
Supplementary Movie M1 describing the processing of samples on the origami paper devices file (windows media video .wmv)
The data associated with this publication is available open access at http://dx.doi.org/10.5525/gla.researchdata.568.

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