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ARTICLE

Paper-based analytical devices for colourimetric detection of *S. aureus*, *E. coli* and their antibiotic resistant strains in milk

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Animal derived milk is an important part of human diets due to its high nutritional value, which not only supports humans but also presents a growth environment for pathogenic bacteria. Milk may become contaminated with bacteria through udder infections or through contact within the dairy farm environment. Infections are treated with antibiotics, with β-lactams most commonly used in veterinary medicine. However, their frequent use leads to the emergence of β-lactam resistant bacterial strains, which causes difficulties for treatment of infections in both humans and animals. Detection of pathogens as well as their antibiotic sensitivity is pre-requisite for successful treatment and this is generally achieved with laboratory-based techniques such as growth inhibition assays, enzyme-linked immunosorbent assays (ELISA) or polymerase chain reaction (PCR), which are unavailable in resource-limited settings. Here, we investigated paper-based analytical devices (µPADs) for the presumptive detection of *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*) and their antibiotic resistant bacterial strains in milk samples. The µPADs were fabricated on filter paper using wax printing, and impregnated with chromogenic substrates, which reacted with bacterial enzyme reacted to form coloured products. Limits of detection of *S. aureus*, *E. coli* and their antibiotic resistant strains in milk samples were found to be 10^6 cfu mL^{-1}. Enrichment of the milk samples in selective medium for 12 h enabled detection to as low as 10 cfu mL^{-1}. The paper devices were tested on a set of 640 milk samples collected from dairy animals in Pakistan demonstrated more than 90% sensitivity and 100% selectivity compared to PCR; suggesting the promise to provide inexpensive and portable diagnostic solutions for pathogenic bacteria in resource-limited settings.

Introduction

Milk is an essential component of human diet and forms part of official nutritional recommendations in many countries. It provides nutrients such as amino acids, vitamins and minerals that are difficult to obtain from dairy-free diets.[1] In addition to being nutritious for humans and animals, milk is also a favourable growth medium for a range of bacteria. Dairy animals can harbour pathogenic bacteria and continuously excrete them in their secretions. Milk can thus become contaminated with pathogenic bacteria either through udder infections, *i.e.* mastitis[2] or via direct contact with contaminated materials.[3] This contaminated milk is a potential source of transmission of foodborne pathogenic bacteria and its nutrient-rich environment allows bacteria to proliferate rapidly and produce toxins, thus making it an extremely vulnerable commodity for human health.[4] In spite of strict quality controls, pasteurization of milk and improved health and well-being of dairy animals in developed countries, outbreaks of milk-borne illness have been reported.[5-7] According to the Center for Disease Control and Prevention (CDC): contaminated milk, and milk products, accounted for the most hospitalizations relating to food-borne illnesses.[8] European Union legislation (Regulation 853/2004) stipulates that only milk from healthy animals can be used for human consumption with a specific limit of <500 cfu mL^{-1} for *S. aureus* and <100 cfu mL^{-1} for *E. coli* (coliforms) in raw milk for drinking (Council Directive 92/46/EEC).[9] US food and Drug Administration suggests lower levels of 10-100 organisms of *S. aureus* and *E. coli* in milk to be of public health concern.[10] Compliance with such legislations can be achieved by constant vigilance over the udder health and the quality of milk until it reaches the consumers. This is particularly challenging in resource-limited countries, where preventive measures are not always followed,[8] and where reliable and affordable point-of-care diagnostic methods are lacking.

Escherichia coli and *Staphylococcus aureus* are major milk-borne pathogenic bacteria that cause a wide range of diseases including mastitis, food poisoning and gastroenteritis in both humans and animals. Mastitis, the most costly and common disease in dairy animals, is mainly treated with beta-lactam

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antibiotics such as penicillin and cephalosporin, typically for more than 3 days.[11, 12] Frequent and prolonged use of antibiotics in feedstock animals is one of the major reasons of the emergence of antibiotic resistant bacterial strains. Beta-lactam resistant strains of both *E. coli* and *S. aureus* have been reported, posing a growing threat in effective treatment of infections related to these pathogens in both humans and animals.[13, 14]

Identification of pathogens and their antibiotic sensitivity is a pre-requisite to tailoring effective prevention and treatment plans. This is usually achieved by cell-culture based methods and growth inhibition assays which are cumbersome, expensive and time-consuming.[15, 16] Alternatively, PCR[16] and ELISA[17] techniques have been used for the detection of these pathogens in milk, but they require the availability of a dedicated laboratory facility, highly trained personnel, stable reagents, and multistep sample handling. Management of the logistical considerations associated with sample collection and transport to central laboratories is also required. Due to the lack of basic infrastructure, these techniques are of limited use in resource-poor settings.[18] According to the World Health Organization (WHO), diagnostic tests for resource-limited environments should be affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free, and deliverable to end-users (ASSURED).[19] More recently, Land *et al.* proposed REASSURED with the addition of R (real-time connectivity) and E (ease of specimen collection and environmental friendliness) to the existing ASSURED, for the design of future diagnostic tests to address important priorities such as global health emergencies and antimicrobial resistance (AMR).[20]

Microfluidic paper-based analytical devices (μPADs) are a relatively new diagnostic platform that may satisfy the ASSURED and REASSURED criteria.[20, 21] Being inexpensive, and simple to manufacture and operate, μPADs have attracted significant interest as emerging alternatives to conventional tests, showing promise for scalable, low-cost monitoring, and user-operated analytical devices. The paper matrix, most commonly hydrophilic cellulose fibres, allows passive liquid transport through capillary forces.[22, 23] Hydrophobic barriers embedded into the fibre allow liquid flow to be confined.[23] A range of methods has been reported for the fabrication of paper-based devices including inkjet printing,[24] photolithography,[21] cutting,[25] stamping,[26] screen printing[27] and wax printing.[28] Wax printing is probably the simplest and most rapid of these fabrication techniques, requiring only a commercially available office 'wax' printer, which operates like an ordinary office printer, but instead of ink, deposits wax on the paper. The printed paper is then heated to allow the wax to penetrate through the paper thickness, and thus generate a hydrophobic barrier in the paper to enable control over fluid flow during an assay.

Recently, there have been reports on μPADs for bacterial diagnostics. Henry's group developed μPADs with colourimetric reaction between bacterial enzymes and chromogenic substrates, *i.e.* chlorophenol red-β-D-galactopyranoside (CPRG) to detect *E. coli* O157:H7, 5-bromo-6-chloro-3-indolyl caprylate for *Salmonella typhimurium*, and 5-bromo-4-chloro-3-indolyl-myo-inositol phosphate for *Listeria monocytogenes* from ready-to-eat meat,[29, 30] and from agricultural water samples.[31] Exploiting the same motif, μPADs were further developed for detection of β-lactam resistance in *E. coli* in water samples; by measuring the colour developed from reaction between nitrocefin substrate and the bacterial secreted β-lactamase.[30] A similar concept was also reported on μPADs for *E. coli* BL21 detection from water samples.[32]

Globally, Pakistan is the fourth largest milk producing country,[33] and *S. aureus* and *E. coli* as well as their antibacterial strains can cause heavy losses to its dairy industry. Early and rapid screening systems can help minimise such losses and also help reducing development of antibiotic resistance for mastitis treatments. The arising Extended Spectrum Beta Lactamases (ESBLs) are not only able to inactivate narrow-spectrum antibiotics such as penicillins, and 1st- and 2nd- generation cephalosporins, but also hydrolyse 3rd, 4th and 5th -generation β-lactam antibiotics such as ceftazidime (CAZ) and cefotaxime (CTX).[34, 35] Infections caused by ESBL-producing bacteria are very difficult to cure, have limited treatment options, and often result in treatment failure.[36, 37] Knowledge of antimicrobial susceptibility patterns of local bacterial population will significantly help clinicians with rapid identification of antibiotic resistant bacterial species, and prescription of appropriate antibiotics in a timely manner.

In this study, μPADs were explored for presumptive diagnosis of *E. coli*, *S. aureus* and their antibiotic-resistant strains in milk samples employing a range of chromogenic substrates (Table 1). For the first time, 5-bromo-6-chloro-3-indolyl phosphate p-toluidine salt (BCIP, Magenta phosphate) and HMRZ-86 were employed on paper-based devices for detection of *S. aureus* and ESBL-positive bacteria, respectively. The developed μPADs were tested with 640 milk samples collected from healthy animals from dairy farms in Pakistan. By combining the various colourimetric assays on the μPADs, a simultaneous indication of presence of particular bacteria and their antibacterial resistance with readout can be achieved by the unaided eye or photographing via smartphone.

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Table 1. Colour changing reactions employed herein to study the presence and antibiotic resistance of *E. coli* and *S. aureus*.

Substrate	Enzyme	Colour change	Indicative	References
CPRG	β-galactosidase	yellow to red-violet	presence of *E. coli*	29, 31
BCIP, Magenta phosphate	alkaline phosphatase	colourless to mauve/purple	presence of *S. aureus*	38
nitrocefin	β-lactamase	yellow to red	antimicrobial resistance to traditional β-lactams	30, 39, 40
HMRZ-86	ESBL	yellow to red	antimicrobial resistance to extended β-lactams	34, 35, 41-43

Experimental

Materials

Bacterial strains of *E. coli* (NCTC 12241, 11560, 13351) and *S. aureus* (NCTC 12981, 12973) were obtained from Pro-Lab Diagnostics (UK). Nitrocefin was obtained from Merck Millipore (UK). Chlorophenol red-β-D-galactopyranoside (CPRG), 5-Bromo-6-chloro-3-indolyl phosphate *p*-toluidine salt (BCIP, Magenta phosphate) or magenta phosphate, β-galactosidase, β-lactamase and rAPid alkaline phosphatase were all acquired from Sigma-Aldrich (UK). HMRZ-86 was purchased from Bio-Rad Laboratories (UK). Filter paper (Whatman grade 4) was bought from GE Healthcare Life Sciences (UK). Muller-Hinton broth and Buffered Peptone Water broth were purchased from Oxoid Ltd., UK. Pasteurised cow's milk was purchased from local stores (Hull, UK).

Preparation of bacterial culture

Pure β-lactamase negative (NCTC 12241), β-lactamase positive (NCTC 11560) and ESBL positive (NCTC 13351) *E. coli* strains were cultured overnight in buffered peptone water (BPW) supplemented with vancomycin hydrochloride (8 mg L^{-1}). Similarly, pure β-lactamase negative (NCTC 12981) and β-lactamase positive (NCTC 12973) *S. aureus* strains were grown overnight in Muller-Hinton (MH) broth supplemented with 5% sodium hydroxide (NaCl). Supplementation of the BPW medium with vancomycin hydrochloride renders the medium selective for *E. coli* while addition of 5% NaCl in MH broth makes the medium selective for *S. aureus.*[31, 44]

Fabrication of μPADs

Designs of the desired wax featured were drawn in AutoCAD software (Student Version 2018). An array of 7 mm diameter circles with 0.5 mm line thickness was printed on the Whatman paper using a wax printer (Xerox ColorQube). The paper was then wrapped in a single layer of aluminium foil and placed on a hot plate at 200 °C for 2-3 min. The heat melted the wax and allowed it to pass through the paper to create a hydrophobic wax barrier. One side of the paper was sealed with masking tape to prevent leaking of reagents during an experiment.

Preparation and deposition of chromogenic substrates in μPADs

Solutions of the chromogenic substrates were prepared in HEPES buffer (0.1 M HEPES, 0.1 % BSA, pH 7.5). CPRG for the detection of *E. coli* was made up as a 3 mM solution according to Bisha *et al.*, 2014.[31] BCIP for the detection of *S. aureus* was prepared at 5.7 mM (the maximum concentration at which the reagent can be dissolved in PBS without precipitation). For the detection of β-lactam resistant strains of *E. coli* and *S. aureus*, a solution of nitrocefin (1 mM) was prepared as described by Boehle *et al.* 2017.[29] HMRZ-86 was available in soluble form in the β-LACTA test kit (Bio-Rad Laboratories) and was used for the detection of ESBL. For a point-of-care device to be used in field conditions, it is desired to have minimal user intervention. To this end, the chromogenic substrates were pre-deposited and dried onto the paper prior to use, in contrast to previous reports where they were added in liquid form.[29, 31] Prior to substrate deposition, the bottom of the printed paper was sealed with masking tape. Then, each chromogenic substrate solution (6 μL) was deposited on the μPADs and allowed to dry at room temperature for 5-10 min (visually inspected). The μPADs were placed in a petri dish covered with aluminium foil to prevent light exposure, and stored at 4 °C for 1-2 days prior to use.

Optimisation of paper-based assays

The colourimetric reactions for paper-based assays were optimised using enzyme solutions prepared in PBS; alkaline phosphatase (0 – 80 mU mL^{-1}), β-lactamase (0 – 0.8 mU mL^{-1}), and β-galactosidase (0 – 80 mU mL^{-1}). The enzyme solutions were freshly prepared at the start of each experiment.

Assays for bacterial culture, spiked milk and real milk samples

Bacterial culture: Ten-fold serial dilutions of *E. coli* and *S. aureus* and their antibiotic resistant strains were prepared in their respective selective enrichment media. Lysis of *E. coli* was performed prior to testing using a probe sonicator (Diagenode Bioruptor/Sonicator UCD-200) at 22 kHz for 30 s to liberate β-galactosidase according to the protocol described by Bisha *et al.*.[31] Alkaline phosphatase and β-lactamase are not endogenous enzymes and therefore, bacterial lysis was not conducted. To perform an assay on a μPAD, 35 μL of pathogen suspension from each serial dilution was added to the pre-prepared μPAD and incubated at 37°C for 3-4 h.

Spiked milk samples: Overnight cultures of *E. coli* and *S. aureus* and their antibiotic resistant strains were used to prepare ten-fold serial dilutions in pasteurized cow's milk (local market, UK). A hundred microlitre of bacterial cell culture suspension was mixed with 900 μL of milk sample. The solution was then further diluted (ten-fold) in milk. For quantification of colony forming units (cfu) per mL, 100 μL from each serial dilution was plated in nutrient agar. Each dilution (35 μL) was tested on the μPAD for colourimetric reaction. Previous studies reported that low volume enrichment (<1 mL)resulted pathogen cell proliferation and an increase in their associated enzymes and therefore a more intense colour change and lower limit of detection were achievable.[29] For this purpose, 900 μL of selective enrichment broth was inoculated with 100 μL of each serially diluted spiked milk sample. These enriched spiked milk samples were incubated at 37 °C for variable periods of time, *i.e.* 0, 4, 8 or 12 h. After incubation, 35 μL from each enriched sample was tested on the μPAD with colourimetric reaction.

Raw milk samples: Following ethical clearance by the Institutional Ethical Committee (IEC) in the National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad (Pakistan), raw milk samples were collected from 640 dairy animals having apparently healthy udders (228 buffaloes and 412 cows in different dairy farms in rural areas of Faisalabad, Pakistan). Before sampling, the initial 3-4 stripes of milk were discarded, and teat ends were disinfected with cotton swabs soaked in 70% ethanol and allowed to dry. Each milk sample (5-10 mL) was collected directly in a sterile screw cap bottle from all quarters. The samples were kept on ice and transported to the laboratory for analysis. For enrichment of bacteria, 1 mL of milk sample was diluted with 9 mL of BPW containing specific supplements and incubated at 37˚C for 12 h (Table S1, ESI). Specific supplement encourages growth of selective bacteria only. After incubation, samples were tested on μPAD for the detection of *S. aureus* and *E. coli* and their β-lactam resistant strains as discussed above.

Visual detection and data analysis

Colourimetric results were analysed through visual inspection of the μPAD at 3-4 h after sample deposition. Presence of *E. coli* in the samples was determined by a change in colour of CPRG embedded in the μPADs from yellow to red-violet.[29, 31] Similarly, *S. aureus* was detected by a change in colour of BCIP containing μPADs from colourless to mauve/purple.[38] A change of colour of nitrocefin[24] and HMRZ-86[35, 41-43, 45] embedded in the μPADs from yellow to red are the indicatives of β-lactamase and ESBL positive bacteria, respectively.

Semi-quantitative analysis of the colourimetric end results was performed according to the previously reported protocol by Boehle *et al*.[24] Briefly, following the assay, petri dishes containing the μPADs were placed inside a light box (16 cm x 16 cm x 16 cm) to avoid light interference during image capture. The camera part of the smart phone (Vodafone 890N, operated with flash) was placed between the slit of the light box (2 cm x 5 cm) to obtain images. The images were analysed using ImageJ freeware (National Institute of Health, USA), following the protocol previously described.[24] Initially, the images were processed through a Red Green Blue (RGB) stack and the green channel was selected as it is the complementary colour of red, which is the end point colour of all reactions except *S. aureus* experiment which was mauve/purple. Although the complementary colour of mauve/purple is yellow, it gave a relatively high sensitivity with green image amongst the RGB stack. Therefore, it was also analysed through the green channel. Next, the image was inverted. Circles were drawn to outline the measurement areas within the reaction zones. Utilising the "Measure" tab under "Analyze", the average colour intensity within the defined areas was obtained and further analysed with Microsoft Excel. The colour intensity of μPADs containing water was also analysed as a blank: the colour intensity of μPADs containing samples was normalized by subtracting the colour intensity of μPADs containing water. Normalised data was processed in Microsoft Excel to obtain mean and standard deviations from three separate repeats (n=3).

UV-vis spectrophotometric detection of colourimetric reactions

UV-vis spectrophotometric analysis of colourimetric reactions employed for μPADs was performed on the same reactions using microtitre plates and a UV-vis plate reader (NanoDrop™ Lite Spectrophotometer, Thermofisher Scientific). For this purpose, 100 μL of ten-fold serial dilutions of *S. aureus*, *E. coli* and their β-lactam resistant bacterial strains were reacted with 100 μL of their respective chromogenic substrates whose concentration were similar to those used on μPADs. The microtiter plate was then incubated at 37 °C for 2 h. Following this, absorbance at various wavelengths were measured; 595 nm for the CPRG reaction, 405 nm for the BCIP reaction, and 490 nm for the nitrocefin and HMRZ-86 reaction.

Polymerase chain reaction (PCR)

DNA was extracted from enrichment broth through GeneJET Genomic DNA Purification Kit according to the manufacturer's instructions. A Nano-drop system (ND-2000C, Thermofisher Scientific) was employed to quantify the concentration and purity of the extracted DNA. Primers used during PCR are summarised in Table S2, ESI. All reactions were carried out to a final volume of 50 μL under the following conditions. Initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation 94 °C for 1 min, annealing (at the temperature summarised in Table S2, ESI) for 1 min, and extension at 72°C for 2 min. Final extension was performed at 72 °C for 10 min to complete the reaction. Amplified PCR products were detected by electrophoresis on 1.5% agarose gel and visualised under gel documentation system (Gel Doc™ EZ Imager, Bio-RAD) and photographed. GeneRuler 1kb DNA ladder (Thermo Scientific) was included in each run.

Results and discussion

Paper-based assays employing colourimetric reactions between bacterial enzymes and their corresponding chromogenic substrates

Development of the paper-based assays initially involved using enzyme solutions, rather than enzymes derived from the bacteria, to verify the feasibility of enzyme-substrate reactions on paper matrix. For optimisation of the reaction conditions different dilutions of pure bacterial enzymes were freshly prepared at the start of each experiment. The lowest β-galactosidase concentration to produce a distinguishable colour change from yellow to red-violet was 0.02 U mL^{-1} (Fig. 1S (a), ESI), comparable to 0.03 U mL^{-1} reported for β-galactosidase.[46] The lowest enzyme concentration to display a distinctive colour change for β-lactamase assay was 0.1 mU mL^{-1} (Fig. 1S (b), ESI). The discrepancy with the value of 10 mU mL^{-1} reported by Boehle *et al.*[24] may plausibly be due to different sources of beta- lactamase employed for the reaction.

For *S. aureus* detection, the reaction between solutions of BCIP and alkaline phosphatase solution was first attempted on paper. Utilising 3 mM BCIP, a characteristic colour change from colourless to mauve/purple was successfully observed at ≥ 0.006 U mL^{-1} of alkaline phosphatase (Fig. 1S (c), ESI). This demonstrates a novel paper-based assay for *S. aureus* detection via the bacterial alkaline phosphatase and its corresponding chromogenic substrate BCIP (Magenta phosphate).

Testing of developed µPADs with bacterial culture

Having successfully developed PADs for detection of bacterial enzymes from enzyme solutions, the devices were next tested with live bacteria populations obtained from cell cultures in their respective growth media. Ten-fold serial dilutions of different strains of *E. coli* and *S. aureus* were prepared in their respective selective broths and reacted with their relevant chromogenic substrate on the µPADs.

In order to determine the presence of β-galactosidase, *E. coli* was lysed prior to testing on the developed paper device as β-galactosidase is produced inside *E. coli* cells and is not secreted into the surrounding medium. A distinctive colour change from yellow to red-violet when reacting with CPRG was observed at *E. coli* concentration ≥ 3.6 x 10^{6} cfu mL^{-1} (Fig. S2 (a), ESI), in good agreement with 3.8 x 10^{6} cfu mL^{-1} value detected on PADs developed by Boehle *et al.*[24]

In contrast to β-galactosidase, alkaline phosphatase is excreted from *S. aureus* cells into the surrounding medium. The reaction between *S. aureus* secreted alkaline phosphatase with BCIP was therefore carried out without cell lysis (Fig. S2 (b), ESI) employing the same device shown in Fig. S1 (c), ESI. A distinguishable colour change from colourless to mauve/purple was observed at concentration exceeding 3.6 x 10^{6} cfu mL^{-1}. To the best of our knowledge, this is the first time the detection of *S. aureus* was demonstrated on a paper device based on the reaction between bacteria secreted enzyme alkaline phosphatase and 5-bromo-6-chloro-3-indolyl phosphate *p*-toluidine salt.

Next, the efficiency of the developed PADs on detecting β-lactam resistant strain of *E. coli* was examined using the colourimetric reaction between nitrocefin and beta-lactamase to produce a characteristic colour change from yellow to red. No cell lysis was performed prior to the reaction, even though a marginal 5% increase in colour intensity on PADs tested with lysed *E. coli* when compared to that of intact cells.[30] As expected, no colour change occurred for the β-lactam susceptible strain of *E. coli* where no β-lactamase was produced by the bacterial enzyme (Fig. S3(a), ESI). For the β-lactam resistant strains, a colour change occurred at concentrations ≥ 10^{6} cfu mL^{-1}, in good agreement with ≥ 3.8 × 10^{6} CFU mL^{-1} bacteria reported from PADs tested with different β-lactam resistant bacteria in wastewater and sewage.[24] The same device subjected to a β-lactam resistant strain of *S. aureus* detected the colour change at ≥ 1.3 x 10^{6} cfu mL^{-1} (Fig. S3(b), ESI).

The experiments confirmed the feasibility of µPADs for *E. coli* and the β-lactam resistant strain of *E. coli*, as well as feasibility for detection of *S. aureus* employing the reaction between their secreted alkaline phosphatase and the chromogenic BCIP substrate. In addition, a β-lactam resistant strain of *S. aureus* was effectively detected using the nitrocefin-embedded µPAD. Distinctive colour changes were observed in all cases at bacterial levels of ≥ 10^{6} cfu mL^{-1}.

Cross reactivity study of µPADs for *S. aureus* and *E. coli*

The colourimetric assays employed for the developed paper devices utilised enzymes which could also be produced by other bacteria, and hence a compromised specificity might be expected when performed under field conditions. In laboratory settings, specific supplements can be added into the enrichment media in order to promote growth of target bacteria, whilst simultaneously inhibiting growth of other competing bacteria. Herein, vancomycin hydrochloride was utilised to support the growth of *E. coli*, and to suppress the growth of *S. aureus*. Similarly, the high salt content present in MH broth only permits *S. aureus* to grow. A cross-reactivity study between the two assays was carried out after incubating the bacteria in selective enrichment broths. The colour change was only observed when the correct pair of bacterial enzyme and specific substrate were present, with no false positive results found from the two bacterial species (Fig. 1). Although cross reactivity can be eliminated in laboratory analysis using growth inhibitor to suppress competing bacteria, this can be problematic in field tests and false positive results are inevitable. Nevertheless, the method can still be suitable as an initial screening test providing impetus for further testing and confirmation to be conducted. Further investigations will include protocols to minimise other related contaminating bacteria in the sample using selective enrichment media.

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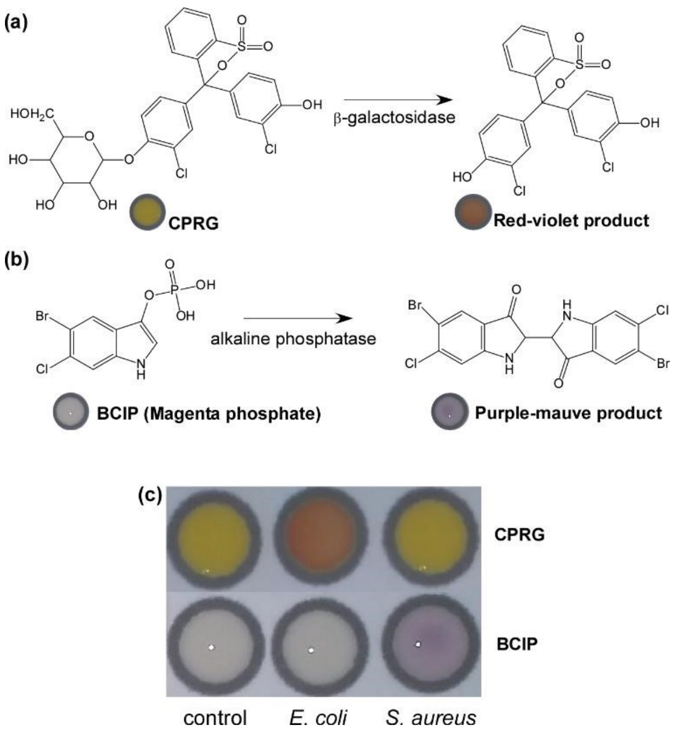

Fig. 1 Cross reactivity study for assessing the selectivity of each enzyme-substrate pair. (a) A reaction between CPRG substrate and the enzyme β-galactosidase from *E. coli* yields a colour change from yellow to red-violet. (b) A purple-mauve colour product is afforded from the reaction between BCIP substrate and the enzyme alkaline phosphatase from *S. aureus*. (c) µPADs embedded with CPRG (top row) and BCIP (bottom row) tested with control (left-hand column), *E. coli* (middle column) and *S. aureus* (right-hand column). Colour change was observed only when a specific substrate-enzyme pair was present. Level of bacteria = 10^6 cfu mL^{-1}.

µPADs for differentiation of β-lactam resistant and ESBL resistant bacteria

Next, we aimed to develop paper-based assays for the detection of ESBL bacteria which do not only show resistance to penicillins, such as cephalosporins and aztreonam, but also to other classes of antibiotics, such as aminoglycosides, cotrimoxazole, tetracycline and fuoroquinolones.

Nitrocefin was reported as a suitable substrate for the detection of ESBL bacteria. A colour change from yellow to red could be observed on the developed µPAD when pathogen concentrations exceeded 10^6 cfu mL^{-1} for both β-lactam resistant *E. coli* and ESBL resistant *E. coli* (Fig. S4 (a), ESI) as nitrocefin cannot differentiate between these two groups.[36] As such, a specific chromogenic cephalosporin, HMRZ-86, was herein exploited as a selective chromogenic substrate embedded on the µPAD to detect ESBL-resistant bacteria. A carboxypropyl-oxyimino group binds to the side chain at position 7 in compound protects the β-lactam ring from a range of β-lactamases, but not from extended-spectrum β-lactamases (ESBLs).[45] In contrast to nitrocefin embedded µPADs, a colour change from yellow to red only occurred from the presence of ESBL-resistant *E. coli* at concentrations exceeding 10^6 cfu mL^{-1} (Fig. S4 (b), ESI). This development can be applied to real samples where differentiation of antibiotic resistant *E. coli* strains can prove to be crucial.

The sensitivity of the paper-based reactions performed on the developed µPADs was validated against the UV-visible spectrophotometry results obtained from the same liquid-phase reactions conducted in conventional microtitre plates. A comparable level of 10^6 cfu mL^{-1} bacteria detection observed from the absorbance values of the spectrophotometric assay confirming the viability of the developed µPADs (Fig. S5-S7, ESI), and demonstrating a cost-effective approach of µPADs coupled with a smart phone for the detection of bacteria in samples without the need of sophisticated laboratory equipment and trained staff.

µPADs for analysis of bacterial spiked-milk samples

Having demonstrated the viability of the paper devices for detection of *E. coli*, *S. aureus* and their antibiotic resistant strains from bacterial culture media, the µPADs were next tested with milk samples spiked with bacteria in a range of ten-fold serial dilutions.

First, the µPAD embedded with BCIP was investigated with milk samples spiked with *S. aureus*. A colour change from the reaction between the *S. aureus* secreted alkaline phosphatase and BCIP was visually detectable at the concentration exceeding 4.5 x 10^6 cfu mL^{-1} (Fig. 2a), similar to the result previously obtained from *S. aureus* in culture media. The nitrocefin reaction also worked well distinguishing between β-lactam susceptible and β-lactam resistant strains of *S. aureus* spiked into milk samples (Fig. 2b). The resistant strain of *S. aureus* revealed a red colour at concentrations exceeding 4.5 x 10^6 cfu mL^{-1}. These results showed successful paper-based detections of *S. aureus* and its β-lactam resistant strain from spiked milk samples, which can potentially be utilised for detecting such pathogenic bacteria from real milk samples and other complex matrices.

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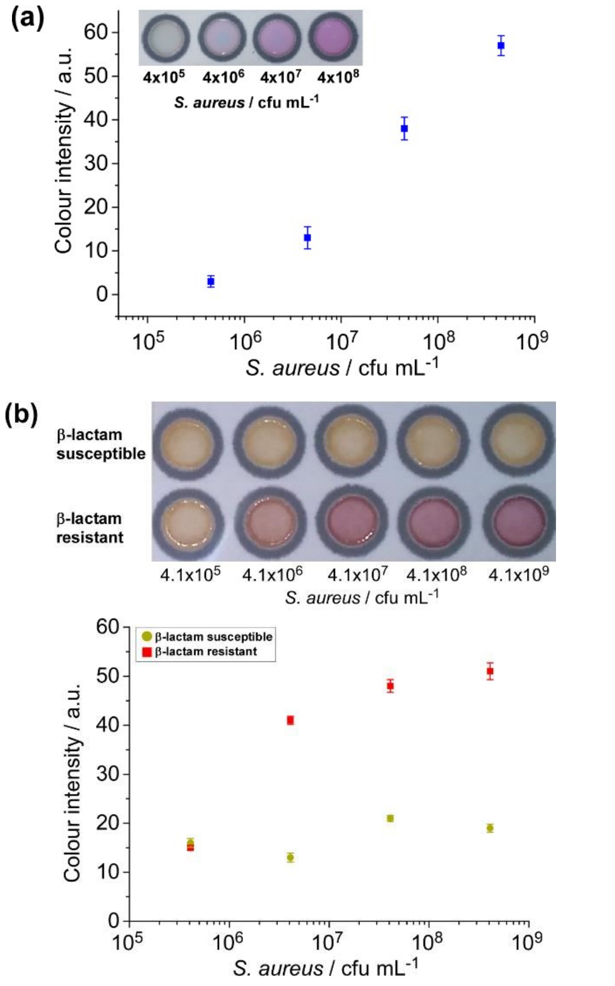

Fig. 2 (a) Reaction on µPAD between the BCIP substrate (5.7 mM) and alkaline phosphatase excreted from *S. aureus* spiked into milk. A colour change was detectable at 4.5 x 10^6 cfu mL^{-1}, no colour change was observed at 4.5 x 10^5 cfu mL^{-1}. (b) Reaction on µPAD between nitrocefin substrate and live bacteria of β-lactam resistant and β-lactam susceptible strains of *S. aureus*. Only the resistant strain yields a colour change when concentrations exceed 10^6 cfu mL^{-1} (n=3).

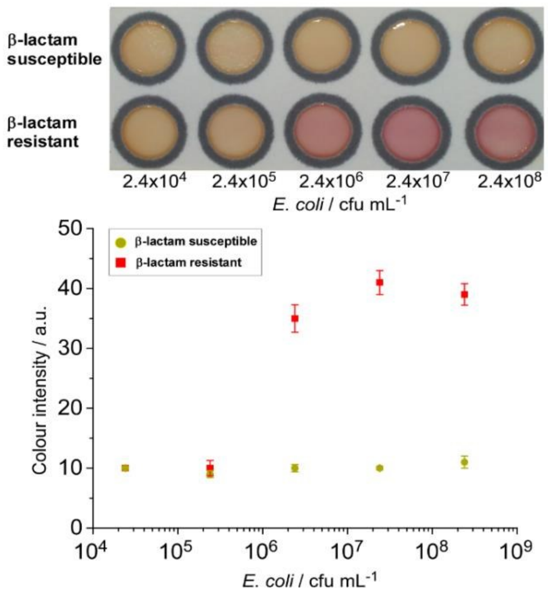

Fig. 3 Reaction on µPAD between nitrocefin substrate and live bacteria of β-lactam resistant and β-lactam susceptible strains of *E. coli.* Only the resistant strain yielded a colour change when concentrations exceeded 10^6 cfu mL^{-1}

The nitrocefin-embedded µPAD tested with *E. coli* spiked into milk samples showed a colour change to red for the resistant strain at concentration ≥2.4 x 10^6 cfu mL^{-1} (Fig. 3), similarly to the observation from the culture broth. Boehle *et al.* also detected a colour change in their nitrocefin embedded µPADs tested with wastewater and sewage with ≥ 3.8×10^6 cfu mL^{-1} bacteria, but not with lower concentrations.[24] This shows the versatility of the paper-based β-lactamase-nitrocefin assays for the detection of β-lactam resistant *E. coli* at ca. 10^6 cfu mL^{-1}, regardless of the complexity of the matrices.

The

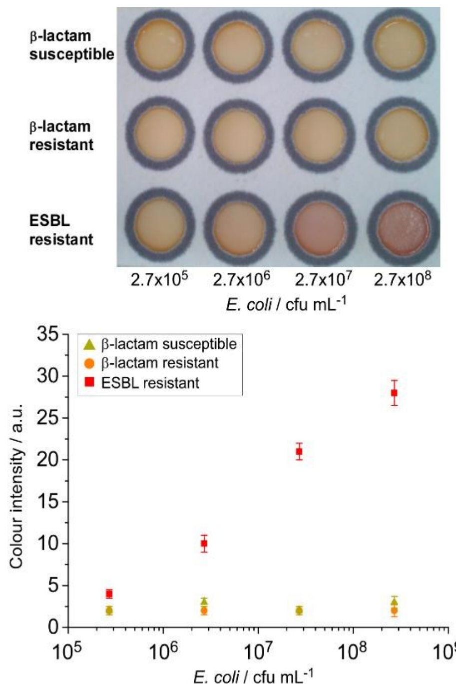

Fig. 4 Detection of ESBL *E. coli* from spiked milk samples employing HMRZ-86 impregnated µPAD. A distinctive colour change from yellow to red was observed from the ESBL resistant strain of *E. coli* at 2.7 x 10^6 cfu mL^{-1} (n=3).

assessment of HMRZ-86 impregnated µPAD with spiked milk samples also demonstrated the specificity to the EMBL-resistant strain of *E. coli* at a similar visual detection level of 2.7x10^6 cfu mL^{-1} as found in pure culture samples (Fig. 4).

However, the reaction between CPRG and β-galactosidase from lysed *E. coli* was not successfully tested with spiked milk samples (Fig. S8, ESI). It is postulated that β-galactosidase released by the bacteria might have hydrolysed a large amount of lactose contained in the milk samples, and therefore only a small quantity of β-galactosidase was available to react with embedded CPRG.

Following on, pre-enrichment of spiked milk samples was attempted in order to supply nutrients and to encourage bacteria growth over a period of a few hours prior to testing. The supplements contained within the enrichment media selectively promote the growth of target bacteria, whilst simultaneously inhibiting the growth of other competing bacteria. Thus, the desired bacteria can be brought to a

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concentration level that is detectable through the colourimetric reaction on the µPADs.

Fig. 5 depicts the colourimetric results obtained from milk samples spiked with *E. coli*, *S. aureus* and their antibiotic resistant strains after pre-enrichment. Levels of bacteria as low as 10 cfu per 1 mL of spiked milk sample could be detected following 12 or 16 h of pre-enrichment in their respective selective broths. This suggests that the sensitivity of the paper-based assays can be vastly improved by including a bacterial pre-enrichment step. Pre-enrichment amplifies the analyte target, and when combined with selective growth media, reduces growth of unwanted bacteria in samples that could contribute to false positive results as also reported previously.[24, 31] This step only required an incubator, which is commonly used in a microbiology laboratories. With further investigations, a suitable pre-concentration step can be included in the platform for field tests. Final detection performed on µPADs can offer a simple, cost-effective, and easy-to-interpret solution to screening of major milk borne pathogens. The entire process, overnight pre-enrichment and colourimetric detection, can be performed within one day, and allows detection of bacterial contamination as low as 10 cfu mL^{-1} from the initial sample.

µPADs for analysis of bacteria in raw milk samples

The ultimate goal for this present study was to develop a simple, cost-effective and user-friendly device for detection of pathogenic bacteria causing mastitis suitable for resource-poor areas in developing countries. The viability of the developed paper devices was therefore examined for detection of *S. aureus* and *E. coli* with raw milk collected from a group of cattle and buffaloes in Pakistan. The samples were collected from healthy animals from the field. Due to very low level of bacteria from the samples, pre-enrichment with selective media was required to increase the number of bacteria to meet with the detectable level of the paper devices (ca. 10^6 cfu mL^{-1}) previously observed from bacterial spiked milk samples. In order to achieve this, a milk sample (10 v%) was mixed with selective media (90 v%) and incubated at 37 °C for 12 h. This step not only increased bacterial level, but also diluted lactose concentration in the samples, thereby diminishing the consequent competing lactose hydrolysis which was problematic in the β-galactosidase-CPRG reaction previously observed in *E. coli* spiked milk samples. Subsequently, the pre-enriched samples were analysed on µPADs and PCR gene analysis for result confirmation (Fig. 6 a,b)).

Out of 640 milk samples, µPADs detected 77 samples with *E. coli* positive (84 samples confirmed by PCR), and 129 samples with
S. aureus positive (143 confirmed by PCR) as summarised in Table 2. These results suggested that the sensitivity of our developed µPADs was higher than 90%. The positive results obtained on µPADs also showed positive from PCR; indicating that the µPADs were also 100% specific.

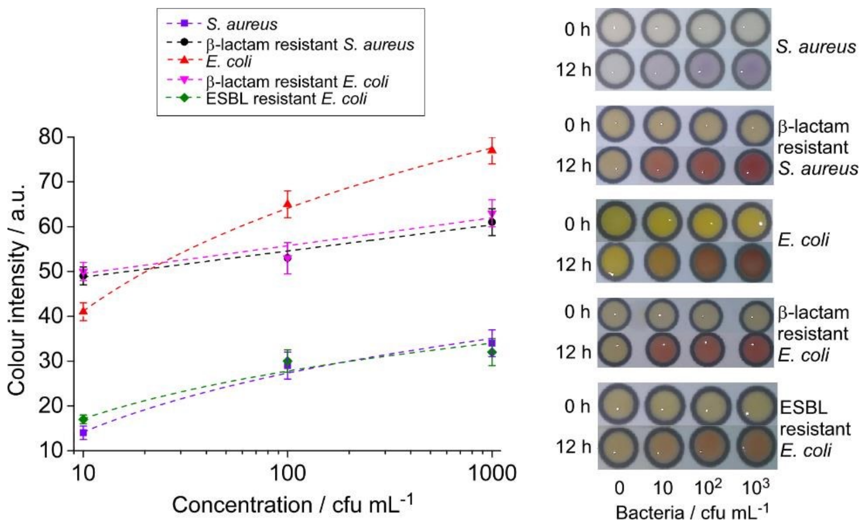

Fig. 5 Photographs and colour intensity analysis of milk samples spiked with various bacteria after 12 h enrichment (n=3).

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Table 2. Comparison of the results from µPADs and PCR for detection of *E. coli* and *S. aureus* from raw milk (n = 640).

Bacteria	Positive samples	
	µPAD	PCR
E. coli	77 (12%)	84 (13.1%)
S. aureus	129 (20.2%)	143 (22.3%)

Beta-lactam is the most widely used class of antibiotics in veterinary medicine. As a result, β-lactam resistant pathogens are very common in livestock. For the detection of β-lactam resistance in the *E. coli*- and *S. aureus*- positive milk samples, enrichment cultures were sub-cultured in BPW supplemented with Cefotaxime (2 µg mL^{-1}) and tested on nitrocefin embedded µPAD. Beta-lactam resistance in the same samples was also confirmed by PCR through amplification of blaTEM gene (Fig. 6 (c, d)). The µPAD detected β-lactam resistant in 67% and 62.7%

of *E. coli* and *S. aureus* isolates, respectively, without false positive confirmed by PCR (Table 3). Therefore, µPAD accurately detected β-lactam resistant isolates.

Table 3. Comparison of the results from µPADs and PCR for detection of *E. coli* and s *S. aureus* from raw milk (n = 640).

Bacteria	Positive samples	
	µPAD	PCR
β-lactam resistant *E. coli*	52/77 (67.5%)	52/77 (67.5%)
β-lactam resistant *S. aureus*	81/129 (62.7%)	81/129 (62.7%)

We have shown a successful implementation of paper-based colourimetric assays for detection of *E. coli, S. aureus* and β-lactam resistant strains in milk samples that could be achieved within a day. The present setup required no sophisticated equipment, and yet yielded similar sensitivity and selectivity to the molecular diagnostic technique of PCR for results obtained from 640 raw milk samples. In developing countries like Pakistan, dairy farms are located in remote and resource limited areas and local veterinary diagnostic laboratories are not equipped with sophisticated equipment like micro-plate readers, thermal cyclers *etc.*, they tend to have only bacterial culture facilities such as incubators and simple microscopes.[1, 47] In these laboratories, routine identification of bacteria and their antibiotic sensitivity is usually carried out by their culture characteristics (less specific) and growth inhibition assays (*i.e.* analysis of bacterial growth in the presence of antimicrobial agents), which give result in 2-4 days.

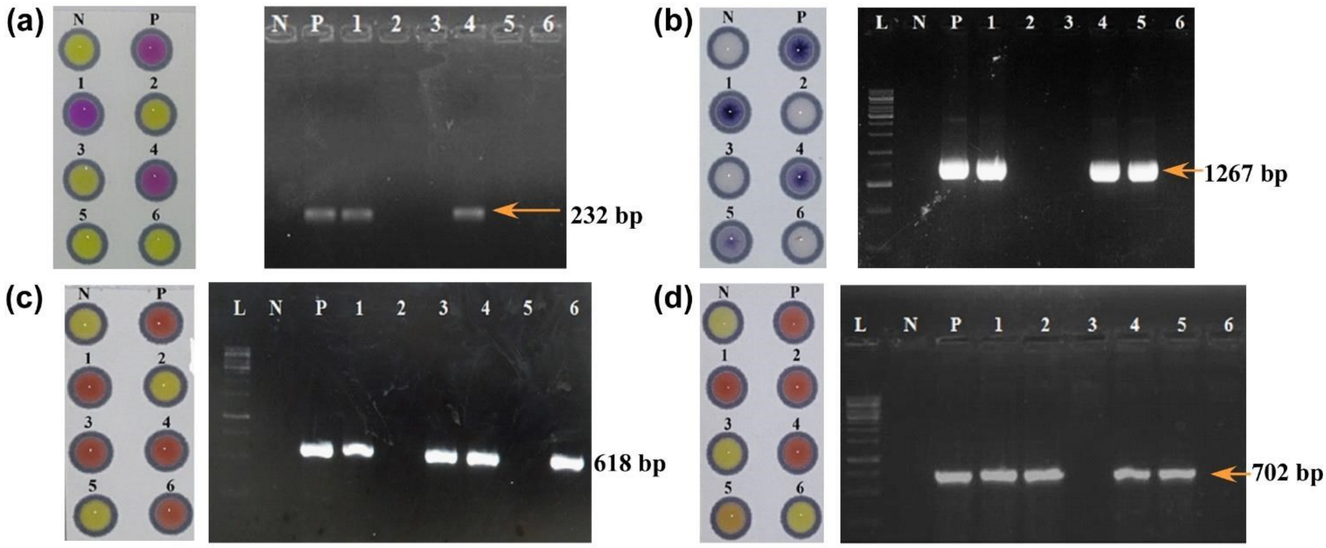

Fig. 6 Left: detection of pathogenic bacteria employing µPADs; (a) *E. coli*. (b) *S. aureus*. (c) Beta-*lactam resistant E. coli*. (d) Beta-lactam resistant *S. aureus*. N = negative control that contains enrichment broth without any sample/bacteria; P= positive control that contains enrichment broth with pathogenic bacteria or their β-lactam resistant strains; 1-6 = samples collected from field. Right: Gel electrophoresis of various PCR products; N = negative control that contains no DNA template; P = positive control that contains DNA of pathogenic bacteria or their β-lactam resistant strains; Lane 1-6 = samples collected from field.

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Conclusions

In the present study, paper-based assays have been devised for the detection of *S. aureus*, *E. coli* and their antibiotic resistant strains in milk samples, with similar sensitivity to the sophisticated laboratory-based technique of UV-vis spectrophotometry. Employing specific chromogenic substrates, colour-change reactions on μPADs have successfully distinguished specific bacteria without cross-reactivity between their β-lactam susceptible and β-lactam resistant strains. They also showed, for the first time, a successful differentiation between ESBL resistant and β-lactam resistant strains. The paper devices obtained ≥90% sensitivity and 100% selectivity, compared to the advanced and sophisticated PCR method, demonstrating a cost-effective platform for the presumptive diagnosis of bacteria in milk especially in resource limited areas.

Conflicts of interest

There are no conflicts to declare.

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References

1. L. Parsons, A. Somoskovi, C. Gutierrez, E. Lee, C. Paramasivan, A. Abimiku, S. Spector, G. Roscigno and J. Nkengasong, *Clin. Microbiol. Rev.*, 2011, **24**, 314-350.
2. S. Oliver, B. Jayarao and R. Almeida, *Foodborne Pathog. Dis.*, 2005, **2**, 115-129.
3. M. Pal, S. Mulu, M. Tekle, S. V. Pintoo and J. Prajapati, *Beverage Food World*, 2016, **43**, 1-3.
4. E. Fox, Y. Jiang and K. Gobius, *Int. Dairy J.*, 2018, **84**, 28-35.
5. D. Morgan, C. Newman, D. Hutchinson, A. Walker, B. Rowe and F. Majid, *Epidemiol. Infect.*, 1993, **111**, 181-187.
6. M. De Buyser, B. Dufour, M. Maire and V. Lafarge, *Int. J. Food Microbiol.*, 2001, **67**, 1-17.
7. D. Schmid, R. Fretz, P. Winter, M. Mann, G. Hoger, A. Stoger, W. Ruppitsch, J. Ladstatter, N. Mayer, A. de Martin and F. Allerberger, *Wien. Klin. Wochenschr.*, 2009, **121**, 125-131.
8. J. Painter, R. Hoekstra, T. Ayers, R. Tauxe, C. Braden, F. Angulo and P. Griffin, *Emerg. Infect. Dis.*, 2013, **19**, 407-415.
9. J. E. Hillerton and E. A. Berry, NMC Annual Meeting Proceedings, 2004.
10. US Food and Drug Administration, Compliance Policy Guide Sec. 527.300, 2012.
11. N. Fejzic, M. Begagic, S. Serie-Haracic and M. Smajlovic, *Bosnian J. Basic Med. Sci.*, 2014, **14**, 155-159.
12. S. Pyorala, *Irish Vet. J.*, 2009, **62**, 40-44.
13. T. Ali, S. Rahman, L. Zhang, M. Shahid, S. Zhang, G. Liu, J. Gao and B. Han, *Front. Microbiol.*, 2016, **7**.
14. B. Robles, D. Nobrega, F. Guimaraes, G. Wanderley and H. Langoni, *Pesquisa Vet. Brasil.,* 2014, **34**, 325-328.
15. J. Jorgensen and M. Ferraro, *Clin. Infect. Dis.*, 2009, **49**, 1749-1755.
16. A. Ashraf, M. Imran, T. Yaqub, M. Tayyab, W. Shehzad and P. Thomson, *Mol. Cell. Probe.*, 2017, **33**, 57-64.
17. R. Bu, J. Wang, C. DebRoy, J. Wu, L. Xi, Y. Liu and Z. Shen, *Iran. J. Vet. Res.*, 2015, **16**, 283-287.
18. C. Rodrigues, N. Desai and H. Fernandes, *Clin. Microbiol. Newsl.,* 2016, **38,** 51-56.
19. G. Wu and M. Zaman, *Bull. World Health Organ.,* 2012, **90**, 914-920.
20. K. Land, D. Boeras, X. Chen, A. Ramsay and R. Peeling, *Nat. Microbiol.*, 2019, **4**, 46-54.
21. A. Martinez, S. Phillips, G. Whitesides and E. Carrilho, *Anal. Chem.*, 2010, **82**, 3-10.
22. D. Liana, B. Raguse, J. Gooding and E. Chow, *Sensors*, 2012, **12**, 11505-11526.
23. T. Satarpai, J. Shiowatana and A. Siripinyanond, *Talanta*, 2016, **154**, 504-510.
24. K. Abe, K. Suzuki and D. Citterio, *Anal. Chem.*, 2008, **80**, 6928-6934.
25. E. Fenton, M. Mascarenas, G. Lopez and S. Sibbett, *ACS Appl. Mate. Interfaces*, 2009, **1**, 124-129.
26. C. Cheng, A. Mazzeo, J. Gong, A. Martinez, S. Phillips, N. Jain and G. Whitesides, *Lab Chip*, 2010, **10**, 3201-3205.
27. Z. Nie, C. Nijhuis, J. Gong, X. Chen, A. Kumachev, A. Martinez, M. Narovlyansky and G. Whitesides, *Lab Chip*, 2010, **10**, 477-483.
28. Y. Lu, W. Shi, L. Jiang, J. Qin and B. Lin, *Electrophoresis*, 2009, **30**, 1497-1500.
29. J. Jokerst, J. Adkins, B. Bisha, M. Mentele, L. Goodridge and C. Henry, *Anal. Chem.*, 2012, **84**, 2900-2907.
30. K. Boehle, J. Gilliand, C. Wheeldon, A. Holder, J. Adkins, B. Geiss, E. Ryan and C. Henry, *Angew. Chem. Int. Ed.*, 2017, **56**, 6886-6890.
31. B. Bisha, J. Adkins, J. Jokerst, J. Chandler, A. Perez-Mendez, S. Coleman, A. Sbodio, T. Suslow, M. Danyluk, C. Henry and L. Goodridge, *Jove-J. Vis. Exp.*, 2014.
32. D. Lin, B. Li, J. Qi, X. Ji, S. Yang, W. Wang and L. Chen, *Sensors Actuat. B-Chem.*, 2020, **303**.
33. M. M. Ajmal, C. X. Li and W. Aslam, *J. Econ. Sustain. Dev.*, 2015, **6**, 19-28.
34. H. Hanaki, Y. Koide, H. Yamazaki, R. Kubo, T. Nakano, K. Atsuda and K. Sunakawa, *J. Infect. Chemother.*, 2007, **13**, 390-395.
35. M. El-Jade, M. Parcina, R. Schmithausen, C. Stein, A. Meilaender, A. Hoerauf, E. Molitor and I. Bekeredjian-Ding, *Plos One*, 2016, **11**.

View Article Online
DOI: 10.1039/D0AN01075H

36. D. Rawat and D. Nair, *J. Glob. Infect. Dis.,* 2010, **2**, 263–274.
37. R. Colodner, *Am. J. Infect. Control*, 2005, **33**, 104-107.
38. US Patent 6,548,268 B1, 2003.
39. S. Bidya and R. S. Suman, *Open J. Clin. Diagnos.*, 2014, **4**, 47-52.
40. S. Sharma, P. Ramnani and J. Virdi, *J. Antimicrob. Chemoth.*, 2004, **54**, 401-405.
41. H. Hanaki, R. Kubo, T. Nakano, M. Kurihara and K. Sunagawa, *J. Antimicrob. Chemoth.*, 2004, **53**, 888-889.
42. S. Jain, J. Andrews, A. Fraise and N. Brenwald, *J. Antimicrob. Chemoth.*, 2007, **60**, 652-654.
43. D. Livermore, M. Warner and S. Mushtaq, *J. Antimicrob. Chemoth.*, 2007, **60**, 1375-1379.
44. M. Bruins, P. Juffer, M. Wolfhagen and G. Ruijs, *J. Clin. Microbiol.*, 2007, **45**, 682-683.
45. H. Hanaki, Y. Koide, H. Yamazaki, R. Kubo, T. Nakano, K. Atsuda and K. Sunakawa, *J. Antimicrob. Chemoth.*, 2007, **13**, 390-395.
46. A. Lokur, *Pharma Innov. J.*, 2018, **7**, 103-106.
47. J. Jacobs, L. Hardy, M. Semret, O. Lunguya, T. Phe, D. Affolabi, C. Yansouni and O. Vandenberg, *Front. Med.*, 2019, **6**.

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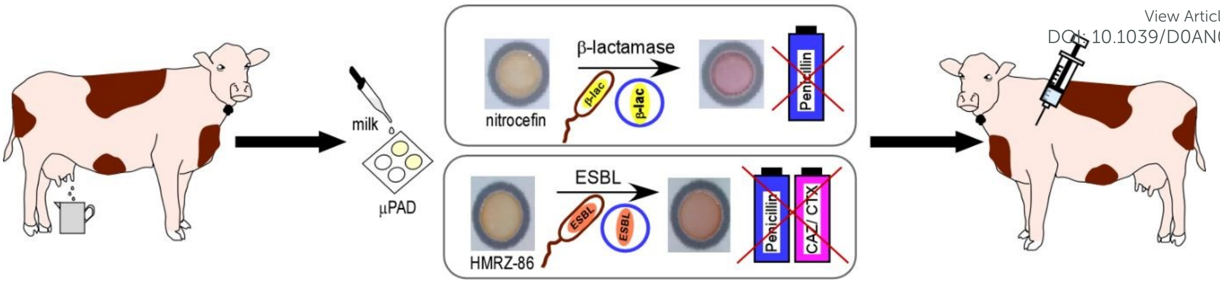
DOI: 10.1039/D0AN01075H



The development of paper-microfluidic devices for the presumptive detection of pathogenic bacteria and their sensitivity towards β-lactamase and Extended Spectrum Beta Lactamases (ESBLs) in milk samples has been investigated to demonstrate a potential inexpensive and portable diagnostic solution for rapid identification of antibiotic resistant bacterial species for appropriate antibiotics prescription for mastitis in a timely manner.