Pen-side diagnostics for bovine respiratory disease

A. Pascual-Garrigos[1,2,3], M. K. Maruthamuthu[1,3], A. Ault[4], J. L. Davidson[1,3], G. Rudakov[1,3,5], D. Pillai[6], J. Koziol[7], J. P. Schoonmaker[8], T. Johnson[8] and M. S. Verma[1,3,5,*]

[1]*Department of Agricultural and Biological Engineering, Purdue University, 225 S University Street, West Lafayette, IN 47907, USA.*

[2]*Department of Biochemistry, Purdue University, 175 South University Street, West Lafayette, IN 47907, USA.*

[3]*Birck Nanotechnology Center, Purdue University, 1205 W State St, West Lafayette, IN, 47907, USA.*

[4]*School of Electrical and Computer Engineering, Purdue University, 465 Northwestern Avenue, West Lafayette, IN 47907, USA*

[5]*Weldon School of Biomedical Engineering, Purdue University, 206 South Martin Jischke Drive, West Lafayette, IN, 47907, USA*

[6]*Department of Comparative Pathobiology, Purdue University, 625 Harrison Street, West Lafayette, IN 47907, USA*

[7]*School of Veterinary Medicine, Texas Tech University, 7671 Evans Drive Amarillo, Texas 79106, USA*

[8]*Department of Animal Sciences, Purdue University, 270 S Russell Street, West Lafayette, IN 47907, USA*

[*]Corresponding author: Mohit S. Verma, msverma@purdue.edu

Abstract

Bovine Respiratory Disease (BRD) is a multi-pathogen syndrome that manifests as a result of stress caused by handling, transport, or an underlying viral infection. At an incidence rate of 18-21%, BRD is the costliest disease in beef and dairy cattle in North America, leading to almost a billion dollars in annual losses. Due to the complexity of the disease, management and therapy are challenging. Despite efforts to advance BRD diagnostics, selected treatment protocols (antibiotics) continue to have a high failure rate. This issue is further complicated by the increasing resistance of BRD pathogens to standard antimicrobial agents. We have developed a pen-side diagnostic assay that detects three different types of bacteria (*Pasteurella multocida, Mannheimia haemolytica,* and *Histophilus somni*) from nasal swabs of cattle. Our assay can be conducted on the farm and provide results in the form of a color change that is visible to the naked eye within an hour. The assay demonstrates 60-100% concordance when compared to lab-based analysis. These assays use loop-mediated isothermal amplification (LAMP) to detect DNA from the sample and thus, can be easily reconfigured for the detection of viruses or antimicrobial resistance genes. They can also be adapted to other diseases such as mastitis or other animals such as pigs or poultry. Widespread use of such pen-side diagnostics can help management decisions for individual animals, reduce the spread of antimicrobial resistance, and increase animal welfare.

Keywords: diagnostics, bovine respiratory disease, loop-mediated isothermal amplification, pen-side

Introduction

Bovine respiratory disease (BRD) is known to be the most common cause of morbidity and mortality in cattle affecting 16% of all beef cattle (USDA, 2013) and causing up to 75% feedlot morbidity in North America (Wilson et al., 2017) and Europe. It is also estimated to cost up to $900 million annually in the beef industry alone (Johnson et al., 2017). This economic burden includes loss in weight of the animals, labor expenses, pharmaceutical costs, and deaths (Griffin et al., 2010). Rapid on-farm diagnostics have the potential to reduce this economic burden because they can supplement the information provide by visual clinical signs and help the veterinarian and producer make appropriate treatment decisions.

BRD is an umbrella term used to describe a condition caused by bacteria, viruses, or co-infection (Taylor et al., 2010; Klima et al., 2019). Generally, it is detected by observing clinical signs such as nasal discharge,

depression, anorexia, cough, and fever (Griffin et al., 2010). However, these clinical signs are insufficient for determining the underlying causative pathogen. Currently, one method for determining which pathogen is causing BRD involves taking a nasal swab sample from the suspected animal and sending it to a diagnostic laboratory, where tests are carried out to identify pathogens (Fulton et al., 2012).

Some of the existing methods used for identifying the pathogen include culturing, enzyme-linked immunosorbent assays (ELISA), electron microscopy, immunohistochemistry, microarrays, and qPCR detection (Fulton et al., 2012; Pansri et al., 2020). Unfortunately, tests of this nature often require specialized equipment, extensive sample preparation and trained scientists making the process costly and lengthy. A mainstay of clinical therapy is the use of broad-spectrum antibiotics, which can have high failure rates (Avra et al., 2017). Antibiotic therapy can fail for several reasons including misdiagnosis, inappropriate drug selection, inappropriate administration rate, dehydration, etc. At the same time, the use of antibiotics is claimed to be the leading cause of antimicrobial resistance, which makes the problem worse over time (CDC, 2013). Thus, diagnostics that identify the pathogen and potentially their antibiotic resistance can help improve treatment by guiding towards targeted antibiotic use.

We have recently designed a loop-mediated isothermal amplification (LAMP) assay to detect the presence of *Pasteurella multocida*, *Mannheimia haemolytica*, and *Histophilus somni* from bovine nasal samples using a fluorescence reporter with 89% analytical specificity and 99% analytical sensitivity (Mohan et al., 2021). In the current work (Pascual-Garrigos et al., 2021), we report a colorimetric assay that can be conducted on the farm to detect these bacterial pathogens (*P. multocida, M. haemolytica, H. somni*). The colorimetric assay has a limit of detection of 1,250 copies of DNA per reaction, an analytical specificity of 100%, and analytical sensitivity in the range of 66.7-100% (when measured using contrived samples). The color change is visible to the naked eye and quantifiable using a camera. We illustrate the functionality of this assay on a feedlot by using a simple water bath based on consumer-grade precision cookers and conducting the assay on nasal samples obtained from five steers. The on-farm results are in 60-100% agreement with PCR assays conducted in the lab on the same samples (Pascual-Garrigos et al., 2021).

Materials and methods

The materials and methods are published in detail recently (Pascual-Garrigos et al., 2021). Here, we summarize them briefly.

Bacterial isolates and mucus sample collection

Glycerol stocks of *P. multocida*, *M. haemolytica*, and *H. somni* isolates were obtained from Purdue University's Indiana Animal Disease Diagnostic Laboratory (ADDL) as previously described (Mohan et al., 2021). Mucus samples were obtained from steers (n=5) approximately 12 months of age and 600 lbs. in weight that had not been given antibiotics for at least 100 days at a feedlot in Indiana (Purdue Animal Care and Use Committee Approval # 1906001911) using rayon-tipped sterile double swabs designed for general specimen laboratory use (BD 220135).

Bacterial DNA isolation and quantification

P. multocida, *M. haemolytica*, and *H. somni* were isolated and final DNA concentrations were quantified according to *Mohan* et al. (Mohan et al., 2021).

Colorimetric quantitative LAMP assay (qLAMP)

The colorimetric assay was conducted by modifying the previously published procedure (Mohan et al., 2021). Specifically, in the colorimetric assay, the New England Biolabs' Warmstart Colorimetric LAMP 2x Master Mix

was used. The mix was coupled with Antarctic Thermolabile uracil DNA glycosylase (UDG) and deoxyuridine triphosphate (dUTP) to minimize carryover contamination throughout the experiment.

Data analysis

Absorbance measurements for each minute at 430, 560, and 620 nm wavelengths were extracted, and the data was normalized using the formula (eq. 1):

$$\text{Colorimetric absorbance ratio} = \frac{\text{ghijkhlmno lp ,+' mqRghijkhlmno lp .*' mq}}{\text{ghikjhlmno lp -*' mqRghijkhlmno lp .*' mq}} \quad (1)$$

The absorbance at 620 nm was used as a baseline, and the 430 nm and 520 nm wavelengths were used to mark the change in color of phenol red from red to yellow. The resulting ratios were plotted against time in Microsoft Excel.

Colorimetric threshold

A one-to-one mixture of pH 7.2 phosphate-buffered saline (PBS, Fisher Scientific 20012050) and pH 8.5 Tris-HCl (Bio Basic SD8141) was prepared. Each condition was added to a 96-well FrameStar® skirted flat optical bottom PCR plate in triplicate, sealed with a PCR film, and inserted into the CLARIOstar Plus to obtain measurements for three minutes. From plotting the data, a colorimetric absorbance ratio was selected as the threshold according to the color changes observed so that colorimetric absorbance ratios above the threshold were considered positive and colorimetric absorbance ratios below the threshold were considered negative.

Primer screening and Limit of Detection (LOD)

Colorimetric absorbance ratios were obtained from qLAMP experiments using a 2x DNA dilution factor (10,000 to 78.125 copies of DNA per reaction). Primers with the highest performance were selected as the best primer sets to detect the bacteria of interest using a python script (Pascual-Garrigos et al., 2021).

Precision cooker experiments (on-farm and in-lab)

LAMP reactions were prepared in individual domed PCR tubes (Thermo Fisher AB0337) using 12.5µl New England Biolabs' Warmstart® Colorimetric LAMP 2x Master Mix, 2.5µl of primer mix, 5µl of DNA free water, and 5µl of mucus sample. An Anova Culinary AN500-US00 Sous Vide Precision Cooker (Amazon B08CF6Y4WF) was filled with water and set to 149°F (65°C). The temperature of the water was verified in the lab using an Hti HT-04 Thermal Imaging Camera (Pascual-Garrigos et al., 2021). The tubes were submerged in the water on the right side (the region with relatively homogenous temperature of 65 °C) either by taping them to the inside of the precision cooker with heat-resistant ¾-inch autoclave tape (Fisher 15904) or by using PCR tube holders designed and 3D-printed in-lab with a Formlabs Form 3B 3D printer using high temperature resin v2 and 0.1 mm layer thickness (Pascual-Garrigos et al., 2021). The tubes were removed from the water after 60 minutes.

The experiment was performed in-lab using the usual procedures to avoid contamination (RNase AWAY™ spray, separation of lab spaces, etc.) and on-farm. For the on-farm experiment, the reagents were prepared in the lab, and the addition of mucus was done on-farm using a 0.5-10 µl single-channel pipette with no additional measures to avoid contamination (Pascual-Garrigos et al., 2021). The mucus addition on-farm happened no more than 30 minutes after extraction from the steers, while the mucus addition in the lab was done 4 days after collecting the samples (the samples being stored at -80°C in the meantime). The samples were stored in water so that the test matrix would be similar in the lab and on the farm.

Images of the tubes were taken at 0 and 60 minutes. Images of the tubes in-lab were taken using the Epson Perfection V800 Photo scanner and images of tubes in-farm were taken using a Samsung Galaxy A50. All images obtained were adjusted by using the white balance tool on Adobe Lightroom to obtain a relatively uniform background. The RGB values of each solution were extracted at 60 minutes using ImageJ and Hue values were calculated to differentiate positive and negative results. Shadows and glows on the images were avoided during this process to increase the accuracy of the results. The Hue scale indicated on a color wheel from 0° to 360°. Red/pink color is around 0-15° and 345-360°, orange and yellow is around 30-60°. Since we set a Hue value of 35 as cut-off (higher than 35 is a positive reaction), the red/pink color on the high end (close to 360 °) was simply set to 0 to avoid confusion.

When comparing the LAMP farm results with PCR, having 2 out of 3 LAMP reactions show the same result as PCR was considered agreement.

Results and discussion

Since the 2000s (Notomi et al., 2000), LAMP has been a widely used method for the detection of pathogens, including bacterial (Mohan et al., 2021; Wang et al., 2023) and viral targets (Davidson et al., 2021; Wang et al., 2021). While a majority of these efforts have been made using fluorescent dyes, there have been some advances in the detection of pathogens by coupling LAMP reagents with dyes that are sensitive to magnesium or pH (Tanner et al., 2015; Davidson et al., 2021). These dyes allow visualization of the result with the naked eye. In the assay presented here, we build on the primers previously designed by us (Mohan et al., 2021) and couple them with a pH-sensitive colorimetric reagent: phenol red (Davidson et al., 2021). The primers were screened through the LOD study. Their analytical sensitivity and specificity were determined by studying their behavior with on-target and off-target DNA mixtures, and the concordance of the reaction results between in-lab and on-farm testing was analyzed (Figure 1).

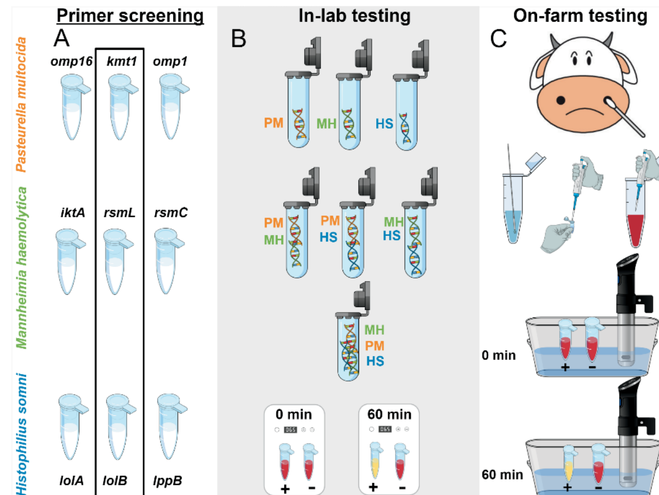

Figure 1: Overall schematic of the workflow. A. Three different primers were screened through the limit of detection (LOD) study. The best selected primers in each species were highlighted inside the black rectangle. B. Several combinations of DNA were diluted in water and tested in the lab environment to study off-target behavior in pH sensitive colorimetric reactions. C. Loop-mediated isothermal amplification (LAMP) was conducted on-farm with a prepared colorimetric master-mix, and later repeated in-lab. A precision cooker was used as a heating device to confirm the ability of our test in a resource limited setting. PM: *Pasteurella multocida,* MH: *Mannheimia haemolytica,* HS: *Histophilus somni* (Pascual-Garrigos et al., 2021).

As seen in previous studies, the Warmstart® LAMP 2x Master Mix, which contains phenol red, is characterized by its transition from pink to yellow as the LAMP reaction occurs and the pH decreases (Davidson et al., 2021; Peltzer et al., 2021). Positive and negative results using our selected primers for the detection of *P. multocida, M. haemolytica, H. somni*, and the pink and yellow distinction can be observed in Figure 2.

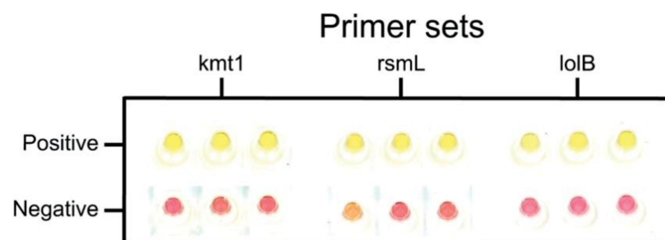

Figure 2: Representative colorimetric results for positive and negative reactions. Positives taken from quantitative loop-mediated isothermal amplification (qLAMP) reactions run with 10,000 copies of DNA per reaction and negatives taken from qLAMP reactions without DNA. Kmt1 primers were used to detect PM, rsmL primers were used to detect MH and lolB primers were used to detect HS. All samples were imaged at 60 minutes. Images were collected using an Epson Perfection V800 Photo scanner and the background was whitened using the ImageJ brightness/contrast setting. PM: *Pasteurella multocida,* MH: *Mannheimia haemolytica,* HS: *Histophilus somni* (Pascual-Garrigos et al., 2021).

In this paper, Table 1 highlights the concordance between conducting the assays on-farm and in-lab. Surprisingly, there is a higher concordance between the on-farm LAMP and in-lab PCR, compared to on-farm and in-lab LAMP. This result suggests that the mucus transportation from the farm to the lab may lead to DNA degradation that leads to false negatives in LAMP reactions (but not in PCR).

Table 1: Concordance between experiments in-lab and on-farm, and between the precision cooker assay on-farm and polymerase chain reaction (PCR). Between loop-mediated isothermal amplification (LAMP) and PCR, 2 out of 3 LAMP reactions with the same result as PCR was considered agreement (Pascual-Garrigos et al., 2021).

Target pathogen	% concordance: precision cooker on-farm vs. in-lab PCR	% concordance: precision cooker on-farm vs. in-lab
P. multocida	100.0%	83.3%
M. haemolytica	60.0%	66.7%
H. somni	100.0%	66.7%

Conclusions

In this work, we developed a colorimetric assay for BRD pathogens with the following six advantages: i) it can be conducted on the farm using a simple consumer-grade water bath, ii) it provides a visual readout and thus, can be analyzed by the naked eye, iii) it provides a response within 60 minutes, iv) it does not require sample processing (e.g., extraction of nucleic acids), v) it can detect the pathogens *P. multocida* and *H. somni* with high accuracy (100% and 96%, respectively), and vi) it utilizes a simple non-invasive nasal swab for sampling.

A major limitation of the current assay is the poor performance of the rsmL primer set for targeting *M. haemolytica.* Even though we performed several screening steps first in our previous work (Mohan et al., 2021) and then in the current work, the primer set had poor performance (accuracy of 79%) mainly due to false negatives. Since the primer set was performing well in pure *M. haemolytica* sample, we speculate that

the drop in performance is due to cross-reactivity with other off-target DNA. We will redesign the primer sets for targeting *M. haemolytica* in future work. Another limitation is the low number (n = 5) of clinical samples tested. Although these numbers are sufficient to demonstrate feasibility of on-farm visual LAMP, they are not sufficient to demonstrate clinical performance. This study serves as a building block for future larger-scale studies.

We anticipate that due to the simple nature of the assay, it can be coupled to the visual observation of animals for clinical signs and help assess the cause of BRD. The assay can determine whether the *P. multocida* and *H. somni* are present in the animals displaying symptoms. The focus of the current work was on demonstrating the feasibility of conducting a visual molecular assay on the farm (instead of the lab). Only detection of BRD pathogens is insufficient for clinical diagnosis in BRD since these pathogens could also be present in healthy animals; thus, we did not evaluate diagnostic specificity, sensitivity, and accuracy. With further development, quantification of these BRD pathogens could help distinguish between healthy and sick animals. Once we include more targets (e.g., *Mycoplasma bovis*, viruses, antimicrobial resistance genes) in our assay, it could also help guide the treatment regimen for BRD.

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