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Loop-Mediated Isothermal Amplification for the Detection of *Pasteurella multocida*, *Mannheimia haemolytica*, and *Histophilus somni* in Bovine Nasal Samples

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Cite This: https://dx.doi.org/10.1021/acsagscitech.0c00072 Read Online

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ABSTRACT: This work develops a loop-mediated isothermal amplification (LAMP) assay that detects the presence of bacterial pathogens (*Pasteurella multocida*, *Mannheimia haemolytica*, and *Histophilus somni*) for bovine respiratory disease complex (BRD) in crude nasal samples. Diagnosing BRD involves a physical examination of cattle expressing physical symptoms linked to the disease. Unfortunately, these symptoms are not unique to BRD alone and do not identify specific pathogens to treat. Nucleic acid-based diagnostics, like polymerase chain reaction (PCR), identify BRD pathogens by amplifying species-specific genes present in samples. However, PCR-based approaches require (i) expensive equipment for successful operation and (ii) DNA extractions to remove PCR inhibitors in samples. LAMP offers an accurate, inhibitor-resistant approach to detecting BRD pathogens in a point-of-care format. This developed LAMP assay is 97% accurate in pure DNA samples, 99% sensitive, and 89% specific in DNA-spiked bovine nasal samples (with 10^4 DNA copies/reaction).

KEYWORDS: *bovine respiratory disease, cattle, animal health, molecular diagnostics, chute-side, nasal swab*

INTRODUCTION

Bovine respiratory disease complex (BRD) is the costliest disease to affect North American beef cattle feedlots with an approximate incidence rate of 18−21%.[1,2] Current methods of detecting or diagnosing pathogenic causes of BRD range from inspection of physical symptoms in cattle (loss of appetite, elevated temperature, and depression) to laboratory assays (serology, cell culture, immunohistochemistry, and in situ hybridization on collected biological materials).[3] However, these methods have drawbacks that can make effective diagnosis and treatment of BRD a problematic endeavor. Physical indicators do not determine the causative pathogen and thus are not sufficient for guiding appropriate therapy. Laboratory-based tests can (i) require lengthy processing times to culture sample pathogens for identification, (ii) require specialized supplies and expensive equipment for accurate performance, and (iii) involve complicated procedures that necessitate trained personnel to operate. Here, we address these limitations by developing a molecular diagnostic assay, using loop-mediated isothermal amplification (LAMP),[4] capable of detecting primary bacterial pathogens for BRD in nasal samples. This work presents novel primers and optimizes them for conducting LAMP for BRD-associated bacterial pathogens.

BRD serves as an umbrella term for a series of respiratory illnesses caused by infections occurring along the respiratory tract.[5] Cattle afflicted with BRD are likely to develop pneumonia, with physical symptoms including elevated temperatures, nasal discharge, depression, and reduced appetite.[6] While treatable, unmanaged cases of BRD can lead to expensive diagnosis/treatments, high morbidity rates, and decreases in overall meat quality.[7,8] Multiple bacteria have been associated with BRD, but the most common are *Pasteurella multocida*, *Mannheimia haemolytica*, *Histophilus somni*, and *Mycoplasma bovis*.[9]

Aside from the assessment of the physical symptoms of cattle to determine BRD disease status, which can often have inconsistent results,[10] the number of diagnostic tests for detecting these BRD pathogens has been growing. These tests include serology, immunohistochemistry, cell culture, and in situ hybridization.[3] While these assays have had nearly three decades of development and standardization, they suffer from requiring sophisticated lab equipment with trained personnel to operate it. Moreover, the influx of livestock samples to test can cause delays between sample submission and assay diagnosis. This bottleneck can result in long wait times for feedlot operators or veterinarians and, more importantly, possible losses of livestock.

Polymerase chain reaction (PCR) offers an approach for detecting BRD by amplifying target DNA sequences that are unique to pathogenic strains.[11,12] However, for many of the same reasons associated with the previously mentioned tests, these PCR assays are restricted to the lab setting. A possible

Received: December 18, 2020
Revised: February 11, 2021
Accepted: February 12, 2021

solution to the equipment limitation is recombinase polymerase amplification (RPA) to detect the presence of BRD pathogens in deep nasopharyngeal swabs.[13] This technique allows for the binding and amplification of target DNA sequences at temperatures of 37−42 °C with recombinase enzymes and DNA binding proteins. RPA is a promising technology and needs to be validated in field settings.

Here, we use LAMP in a manner similar to that of RPA. LAMP is a promising technology for the detection of infectious agents from multiple biological sources.[14] LAMP overcomes the restrictions of other diagnostic methods by providing the following four advantages: (i) amplifies DNA under a single-temperature incubation of ∼65 °C, (ii) improves assay specificity due to the use of four to six DNA primers (which can be designed using freely available software PrimerExplorer), (iii) achieves a limit of detection similar to that of conventional PCR, and (iv) requires only a simple heating element for assay operation as opposed to complex thermocyclers.[4]

Here, we design and characterize a LAMP assay that can be used to specifically target and detect the presence of *P. multocida*, *M. haemolytica*, and *H. somni* from minimally processed bovine nasal samples.

MATERIALS AND METHODS

Design of LAMP Primers for BRD Pathogens. We searched the literature for highly conserved genes present in individual BRD pathogens, and candidate gene sequences were run through the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI). Any species-specific genes that were ≤50% similar with other pathogen genomes were considered unique gene targets and used as template sequences for LAMP primer design (Table 1). Additionally, all available genomes of a single BRD pathogen in the BLAST database were aligned, and gene sequences that were conserved among these alignments were manually determined (>99% similar). The chosen genes were then compared to genomes of other tested BRD pathogens to ensure species specificity by using the ≤50% similarity criterion.

Table 1. Candidate Gene Targets for BRD Bacterial Pathogens

bacterium	candidate gene name	sequence/GenBank ID
P. multocida	*kmt1*	AF016259.1
	ompP1	QGV32322.1
	omp16	AJ271673
M. haemolytica	*rsmL*	QEC27547.1
	rsmC	QEC27614.1
	lktA	QEC25656.1
H. somni	*lolA*	ACA31013.1
	lolB	ACA31225.1
	lppB	ACA32113.1

Three unique gene targets were chosen for each BRD pathogen to verify target pathogen identification for the final assay. Three different primer sets were designed for each gene target to conduct preliminary screening and optimize reaction performance.

All LAMP primer sets were generated using Primer Explorer V5 (http://primerexplorer.jp/lampv5e/index.html). Primer sets that spanned ≤200 bp of a target gene sequence, had 18−21 bp loop primers, and had d*G* values of no more than −4.0 kcal/mol for (i) the 3′ end of F2, (ii) the 5′ end of F1c, (iii) the 3′ end of B2, and (iv) the 5′ end of B1c were selected for initial screening. For each gene target, a total of three different LAMP primer sets were designed.

Bacterial Isolates and Complex Sample Collection. Pure isolates of *P. multocida*, *M. haemolytica*, and *H. somni* were acquired in the form of glycerol stocks from the Indiana Animal Disease Diagnostic Laboratory (ADDL) at Purdue University. Nasal swabs were collected from 45 healthy heifers at the Purdue Animal Sciences Research and Education Center Beef Unit (Purdue Animal Care and Use Committee Approval 1906001911) using rayon-tipped polyester swabs with Liquid Amies transport medium (BD 220146). All nasal samples were then pooled, vortexed until homogenous, and aliquoted for use as a complex substrate for cross-reactivity studies.

Bacterial DNA Extraction. *P. multocida* and *M. haemolytica* isolates were streaked on tryptic soy agar plates supplemented with defibrinated sheep blood (blood agar) and aerobically incubated for 16−18 h at 37 °C. Single, isolated colonies of *P. multocida* and *M. haemolytica* were picked from plates, inoculated into brain−heart infusion (BHI) broth, and incubated aerobically at 37 °C for 16−18 h. *H. somni* isolates were similarly streaked on blood agar plates, stored in GasPak EZ container systems (BD 260672) with BD BBL CO_2 gas generators (BD 260679) from BD (Franklin Lakes, NJ), and incubated in a 5% CO_2 atmosphere at 37 °C for 2−3 days or until sufficient colony growth was present. *H. somni* colonies were inoculated into tryptic soy broth (TSB), stored in the previously mentioned BD GasPak EZ container system with the CO_2 gas generators, and incubated with 5% CO_2 at 37 °C for 2−3 days.

Genomic DNA of all bacterial isolates was extracted from 1−2 mL of the saturated liquid culture using the PureLink Genomic DNA Mini Kit from Invitrogen (Carlsbad, CA) with a final eluted volume of 30 μL. Final DNA concentrations (nanograms per microliter) of eluted extracts were measured using the Quant-iT PicoGreen dsDNA Assay Kit from Invitrogen.

Bacterial DNA Verification. PCRs were conducted on extracts using gene-specific target primers and run on 2% agarose gels to confirm bacterial genome identity. Gels were 2% (w/v) agarose and were run in a horizontal electrophoresis chamber in Tris Borate EDTA for 60 min at a voltage of 80 V. The bands were seen at expected locations as confirmed by a 1 kb ladder (gel images not shown).

Quantitative LAMP Assay (qLAMP). LAMP reactions were conducted by following the manufacturer's instructions of the Warmstart LAMP Kit (DNA & RNA) from New England Biolabs (Ipswich, MA). Reaction mixtures (25 μL) comprised 12.5 μL of Warmstart LAMP 2× Master Mix [40 mM Tris-HCl, 20 mM $(NH_4)_2SO_4$, 100 mM KCl, 16 mM $MgSO_4$, 2.8 mM dNTPs, 0.28 μM dUTP, 0.64 unit/μL Warmstart Bst 2.0 DNA polymerase, 0.6 unit/μL Warmstart Reverse Transcriptase (RTx), 4×10^{-4} unit/μL Antarctic Thermolabile UDG, and 0.2% Tween 20 (pH 8.8 and 25 °C)], 2.5 μL of a 10× LAMP primer mixture (2 μM F3, 2 μM B3, 4 μM LF, 4 μM LB, 16 μM FIP, and 16 μM BIP), 5 μL of a 1:101 dilution of the included LAMP fluorescent dye, and 5 μL of the template DNA-containing solution. Antarctic Thermolabile UDG and dUTP were added to the LAMP reaction mixture for the limit of detection and complex cross-reactivity studies to minimize carryover contamination during assay preparation. In-house validation experiments have confirmed that UDG and UTP do not affect reaction performance at the concentration used. Unless specified, the final concentration of template DNA for qLAMP reactions was 1 ng/reaction. Reaction mixtures were pipetted into wells of white 96-well full-skirted PCR plates from Thermo Fisher Scientific (Waltham, MA). Wells were sealed with VersiCap Mat Cap Strips from Thermo Fisher Scientific and inserted into either a CFX96 Touch Real-Time PCR Detection System from Bio-Rad (Hercules, CA) or a qTOWER^{3}G from Analytik Jena (Jena, Germany) for real-time fluorescence measurement. Reaction plates were incubated at 65 °C for 1 h with fluorescence measurements taken using the FAM/SYBR Green I filter every minute. Ramp rates of 6 and 8 °C/s were used on the CFX96 and qTOWER^{3}G systems, respectively. A ramp rate of 0.1 °C/s was used on the qTOWER^{3}G for the limit of detection and complex reactivity experiments to improve the overall limit of detection of the LAMP reactions.

https://dx.doi.org/10.1021/acsagscitech.0c00072
ACS Agric. Sci. Technol. XXXX, XXX, XXX−XXX

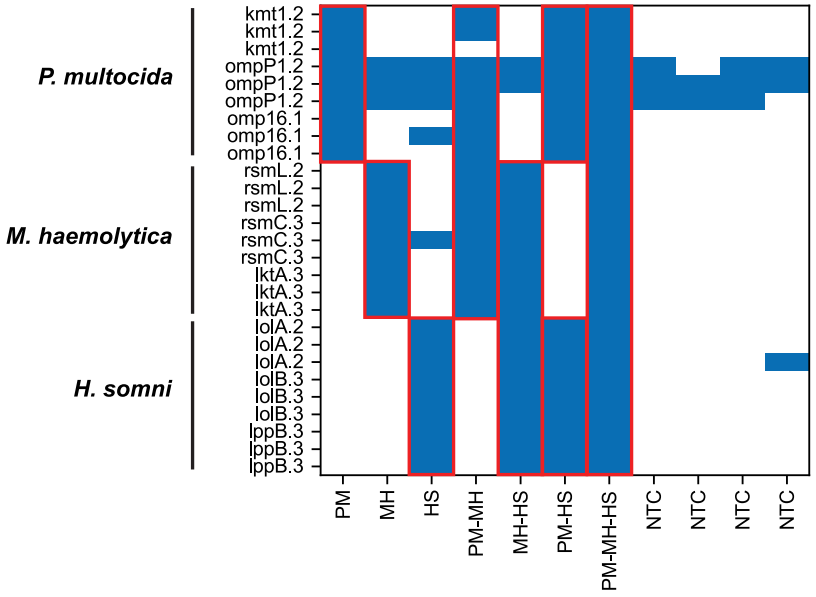

Figure 1. Classification table of selected primers tested against pooled bovine nasal samples with combinations of spiked-in bacterial genomic DNA (gDNA). Final concentrations of spiked DNA in all reactions were 10^4 copies/reaction. Loop-mediated isothermal amplification (LAMP) reactions were conducted in a real-time thermal cycler at 65 °C. Calculated Tt values of reactions were compared to primer set-specific Tt thresholds established from limit of detection (LoD) studies to determine positive vs negative reactions. Reactions that had Tt values less than or equal to the Tt threshold were considered positive reactions and plotted in blue in the classification table. All primer reactions were run in triplicate, and a base bovine nasal sample spiked with water was used as a no-template control (NTC). Outlined red regions represent expected regions of positive reactions. Abbreviations: PM, *P. multocida*; MH, *M. haemolytica*; HS, *H. somni*.

To minimize false positives due to amplicon aerosol contamination, preparation of LAMP reaction mixtures, template DNA loading, and incubation and measurement of reaction mixtures were conducted in separate lab spaces. RNase AWAY Surface Decontaminant from Thermo Fisher Scientific was thoroughly applied to all working surfaces, reagent containers, pipettes, and lab gloves before and after each lab space operation, and the equipment wiped completely with Kimwipes to prevent residue formation. Care was taken in the following three ways: (i) minimize plate agitation during reaction preparation and DNA loading, (ii) securely depress cap strips to wells before and after assay steps, and (iii) wrap plates with aluminum foil (cleaned with RNase AWAY) for transport between lab spaces.

Data Analysis and Figure Generation. Collected fluorescence data from real-time thermal cyclers were exported as Microsoft Excel worksheets (.xlsx) and manipulated in Microsoft Excel or custom MathWorks MATLAB scripts (see the Supporting Information).

Primer Screening. We used five metrics to characterize the primer performance based on their amplification curves: (i) response time, (ii) response time spread, (iii) maximum fluorescence intensity, (iv) maximum intensity spread, and (v) total false positives. Response time was based on the time point at which 90% of the maximum reaction intensity occurs. False positive reactions were defined as reactions with negative/nontarget controls that had fluorescence intensities that were >20% of the maximum reaction intensity. Individual performance characteristic data for all screened primer sets were normalized and multiplied by predefined numerical weights to generate individual characteristic scores for each primer set. All individual metric scores for a single primer set were then summed to generate a total performance score. Any primer sets that produced nontarget amplification in <30 min were automatically rejected and given a total performance score of 0.

Multi-Isolate/Cross-Reactivity Data. Multi-isolate data refer to qLAMP assays run on genomic DNA (gDNA) from different strains (isolates) of *P. multocida*, *M. haemolytica*, and *H. somni*. Cross-reactivity data refer to qLAMP assays run on different gDNA combinations of single isolates of *P. multocida*, *M. haemolytica*, and *H. somni*. For multi-isolate data, fluorescence intensities were extracted for the 30 min time point for all primer set reaction replicates and arranged in a table ordered by isolate. Table values were converted to a heat map and formatted using OriginLab OriginPro to display amplification differences between primer sets for all isolates visually. For cross-reactivity data, fluorescence intensities were normalized and used to find reaction Tt values (time required for the intensity to reach or exceed the defined reaction threshold) for each reaction replicate. These Tt values were then compared to Tt thresholds determined from limit of detection studies to classify reaction replicates as positive and negative reactions. These classifications were classified as a table ordered by spike-in combination.

Receiver Operator Characteristic Curve. Receiver operator characteristic curves were generated by comparing formatted multi-isolate data and cross-reactivity to a predefined threshold via binary classifications to assess positive versus negative reactions. Thresholds were defined as a percentage of the maximum fluorescence intensity of the data set. Various thresholds (0−100%) at increments of 1% were tested and used to calculate the true positive rate and false positive rate for each threshold classification. Diagnostic sensitivity and specificity of the LAMP assay to multi-isolate data were defined as the true positive rate (eq 1) and one minus false positive rate (eq 2) for the lowest threshold value that created the most significant difference in sensitivity between the receiver operator characteristic (ROC) curve and the random chance line. Accuracy was determining by taking the area under the ROC curve.

$$\text{true positive rate (TPR)} = \frac{\text{no. of true positives}}{\text{no. of true positives} + \text{no. of false negatives}} \quad (1)$$

$$\text{false positive rate (FPR)} = \frac{\text{no. of false positives}}{\text{no. of false positives} + \text{no. of true negatives}} \quad (2)$$

Limit of Detection. Fluorescence intensities were extracted for the 45 min time point for all primer set reactions. Intensities were normalized and multiplied by 100 to represent a percent amplification value. Any amplification values that were greater than the previously determined ROC threshold (percent amplification) were highlighted in light blue and considered successful amplification. The lowest DNA

https://dx.doi.org/10.1021/acsagscitech.0c00072
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Table 2. Limits of Detection of Assay Primer Sets[a]

DNA Concentration (copies/reaction)	*P. multocida*																	
	kmt1.2						ompP1.2						omp16.1					
	Water			Liquid Amies			Water			Liquid Amies			Water			Liquid Amies		
1.00E+05	60.56	57.55	54.79	61.89	67.05	70.77	81.57	79.6	81.86	74.33	75.14	73.42	81.11	79.5	77.84	66.15	66.35	59.27
1.00E+04	71.64	77.09	76.37	75.53	69.27	76.16	93.37	100	87.69	69.6	76.94	73	86.53	79.42	84.01	66.49	65.44	61.41
1.00E+03	80.67	77.15	81.19	0.8	87.19	0.06	84.01	84.12	94.94	0.4	0.3	69.59	68.76	77.72	78.27	0.15	0.42	47.03
1.00E+02	-0.36	83.9	89.71	0.73	0.12	1.25	76.05	89.93	-0.66	0.29	0.52	0.64	-0.44	-0.56	-0.43	0.4	25.03	0.43
1.00E+01	-0.47	-0.21	-0.32	0.77	0.64	0.16	-0.39	-0.26	-0.47	0.61	0.57	0.66	-0.12	0.02	-0.06	0.5	0.68	0.37
1.00E+00	0.02	-0.63	-0.38	0.73	0.55	0.48	0.76	-0.54	-0.7	0.26	0.29	0.32	-0.69	-0.65	10.77	0.53	0.47	0.34

DNA Concentration (copies/reaction)	*M. haemolytica*																	
	rsmL.2						rsmC.3						lktA.3					
	Water			Liquid Amies			Water			Liquid Amies			Water			Liquid Amies		
1.00E+05	34.38	32.16	33.32	31.33	33.12	36.57	42.34	39.5	38.01	32.71	31.23	31.63	39.14	38.14	38.15	32.6	35.98	37.67
1.00E+04	38.96	36.95	37.71	33.92	33.06	34.06	39.21	40.98	39.84	32.33	33.49	33.91	36.68	42.45	38.52	33.89	34.02	36.32
1.00E+03	41.42	42.22	43.63	32.46	34.08	32.12	39.36	40.56	37.43	11.11	0.3	24.3	40.79	42.45	41.09	34.47	35.38	36.14
1.00E+02	36.69	36.77	40.81	0.38	0.38	26.32	25.93	0.1	-0.31	0.54	0.48	0.54	30.16	42.1	27.6	0.03	0.46	31.77
1.00E+01	-0.53	0.04	-0.42	0.35	0.35	0.24	-0.3	-0.08	-0.17	0.69	0.54	0.84	32.04	30.51	31.12	1.8	0.03	0.05
1.00E+00	-0.49	-0.23	-0.54	0.2	0.22	0.13	-0.29	-0.54	-0.46	0.62	0.53	0.38	28.8	29.54	31.28	0.15	9.15	0.66

DNA Concentration (copies/reaction)	*H. somni*																	
	lolA.2						lolB.3						lppB.3					
	Water			Liquid Amies			Water			Liquid Amies			Water			Liquid Amies		
1.00E+05	75.54	71.39	76.26	61.24	62.34	64.67	85.2	79.31	78.4	70.94	58.13	65.37	75.47	70	73.4	55.88	57.32	54.26
1.00E+04	73	70	73.87	62.91	64.24	63.94	92.57	89.26	93.93	70.29	65.47	73.8	69.57	71.08	68.48	60.75	60.01	54.66
1.00E+03	73.35	62.74	76.67	66.82	67.12	63.03	76.72	83.23	86	0.67	0.99	-0.8	67.04	64.7	63.4	12.43	0.06	0.06
1.00E+02	0.04	-0.44	5.46	12.81	0.37	16.33	-0.54	-0.35	-0.39	-1.06	-0.86	-0.83	0.28	0.11	0	-0.13	0.23	1.63
1.00E+01	-0.2	-0.51	-0.27	0.7	0.41	0.2	-0.46	-0.33	-0.34	-0.97	-0.68	-0.58	0.28	0.26	0.3	0.48	0.06	0.14
1.00E+00	-0.31	-0.35	-0.47	0.69	0.51	0.46	-0.73	0.64	-0.55	-1	-0.63	-0.72	0.53	0.19	0.03	0.42	0.18	0

[a]Serial dilutions (1×10^0 to 1×10^5 copies/reaction) of genomic DNA (5 μL) were added to reaction mixtures (20 μL of reagents) in triplicate and incubated for 60 min at 65 °C. Fluorescence intensities of primer set replicates at 45 min were extracted and are shown in the table. These values were then compared to a threshold fluorescence intensity (28%) determined from receiver operator characteristic (ROC) analysis. Blue highlighting denotes reactions greater than the 28% threshold should be considered as positive. The lowest concentration at which all three replicates are amplified is the limit of detection. lktA.3 seems to form dimers in water and lead to false amplification, which is inhibited in Liquid Amies medium.

concentrations that had successful amplification for all three replicates of a given primer set were classified as the limit of detection (LoD) for the primer set. Tt value thresholds for each primer set were determined as the time when all amplification fluorescence values at the complex sample LoD were greater than or equal to the ROC threshold.

RESULTS AND DISCUSSION

Assessing Primer Performance in Complex Bovine Nasal Samples. LAMP reactions with optimal primer sets were conducted on pooled bovine nasal swabs in Liquid Amies to determine assay performance with complex samples. Due to the lack of established literature on quantifiable concentrations of BRD bacterial pathogens in sick bovine nasal swabs, pathogen gDNA was spiked into complex samples at a level equivalent to 10^4 copies/reaction to simulate elevated levels of bacteria for BRD-related infection. Different combinations of bacterial pathogen gDNA were spiked into complex samples to reflect possible bacterial communities present in collected samples. Most primer sets could amplify spiked target DNA regardless of the combination (Figure 1). The diagnostic sensitivity and specificity for the assay based on ROC analysis of the cross-reactivity data were 99% and 89%, respectively.

Off-target amplification was observed for the *P. multocida* ompP1.2 primer set in every combination of the spiked complex samples, including negative controls. Further PCR conducted on the base complex sample used for spike-in (Figure S1) revealed DNA sequences encoding the kmt1 gene present in *P. multocida*. This suggests the presence of naturally occurring *P. multocida* in the original swab samples that could contribute to high background amplification in non-*P. multocida* target LAMP assays. However, the significantly higher false positive rate for ompP1.2 in comparison to those of other *P. multocida* LAMP primer sets could indicate a larger DNA load of the ompP1 gene or primer-specific cross-reactivity. Further analysis of *P. multocida* primer set Tt values (Figure S2) shows a shorter range of discrimination between expected positive and negative reactions in ompP1.2 when compared to other *P. multocida* primer sets. In future studies, dropping the ompP1.2 primer set from the overall LAMP assay, due to this narrow discrimination window, would improve the overall assay performance. While one false negative reaction occurs for kmt1.2 in the "PM+MH" column, the calculated Tt value for this reaction was larger than the threshold by merely 2 min. Further replicates of these complex sample reactions would provide better tolerance values for Tt when determining positive versus negative reactions.

To date, no reported PCR assays have been capable of detecting the presence of BRD bacterial pathogens in crude bovine nasal samples. PCR assays experience significant inhibition in the presence of transport media; therefore,

https://dx.doi.org/10.1021/acsagscitech.0c00072
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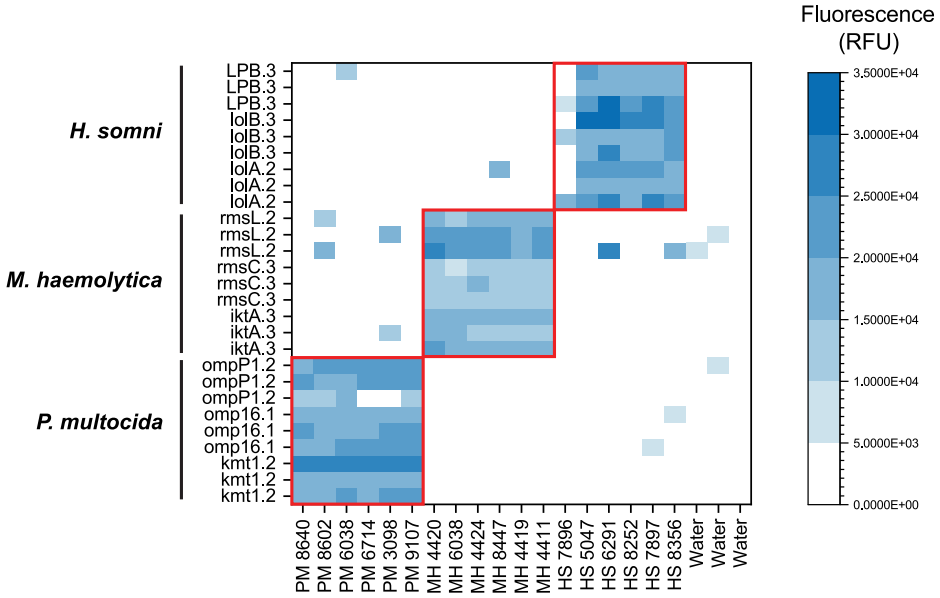

Figure 2. Heat map of selected primer sets tested in triplicate (*y*-axis) against different isolates (six) of BRD pathogens (multi-isolate data). Isolates of *P. multocida*, *M. haemolytica*, and *H. somni* were used (the initials on the *x*-axis refer to the bacterial genus and species, and the numbers refer to a different strain as labeled by the Indiana Animal Disease Diagnostic Laboratory). Loop-mediated isothermal amplification (LAMP) reactions were run in real time with a real-time thermal cycler at 65 °C, and fluorescence intensities were selected at 30 min (longest reaction time of nine selected primer sets) to be plotted on the heat map. Three replicates of each reaction were run and are displayed individually on the map. Water was used as a negative control. Outlined red regions represent expected regions of positive reactions.

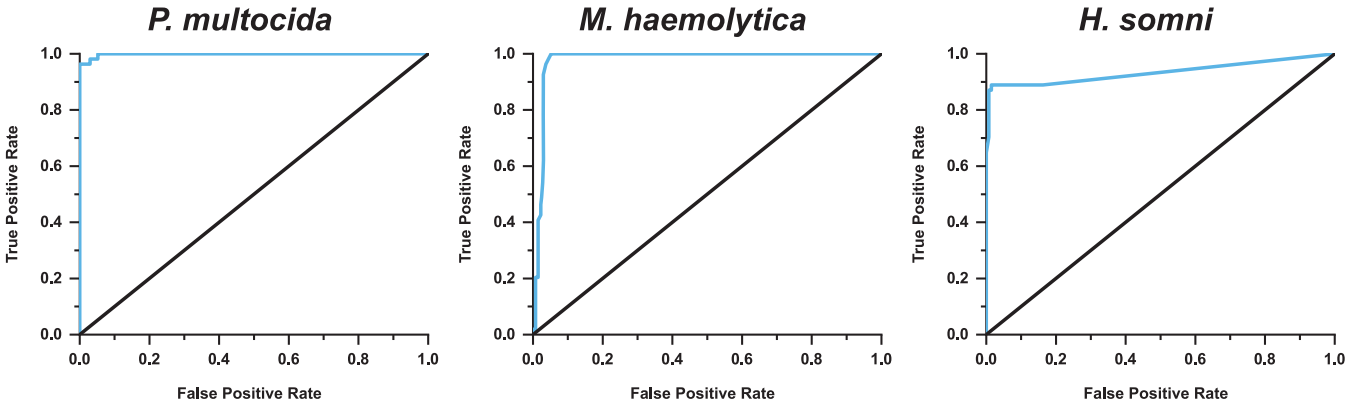

Figure 3. Receiver operator characteristic (ROC) curves illustrating the true positive rate (TPR) and false positive rate (FPR) of the BRD LAMP assay for each pathogen using multi-isolate data presented in Figure 2.

Table 3. Diagnostic Sensitivities and Specificities of LAMP and qPCR Assays against Listed BRD Pathogens

	sensitivity (%)			specificity (%)		
pathogen	LAMP (this work)	qPCR[17]	qPCR[18]	LAMP (this work)	qPCR[17]	qPCR[18]
P. multocida	96	85	84	100	69	70
M. haemolytica	100	72	92	95	91	73
H. somni	89	85	100	99	84	76

some form of DNA purification step before detection can be required.[15,16] As such, our LAMP assay represents a novel approach for screening BRD bacterial pathogens in crude bovine nasal samples with high sensitivity and specificity.

Determination of the Assay Limit of Detection in Simple and Complex Samples. Optimal primer sets were characterized using LAMP reactions with decreasing concentrations of the target gDNA template to assess limits of detection (LoDs), defined as the lowest concentration at which 3/3 replicates show amplification. LoD experiments were conducted on target gDNA suspended in water and Liquid Amies separately to determine inhibitory effects on reaction performance. The performance of our optimal primer sets is highlighted in Table 2. LoDs were predominately (i) 10^3 copies/reaction in water samples and (ii) 10^4 copies/reaction in Liquid Amies samples. This order of magnitude difference in LoD between the two types of media was likely due to the LAMP reaction composition being altered by the increased salt concentrations present in Liquid Amies, negatively impacting reaction sensitivity. As the DNA concentration decreases, there

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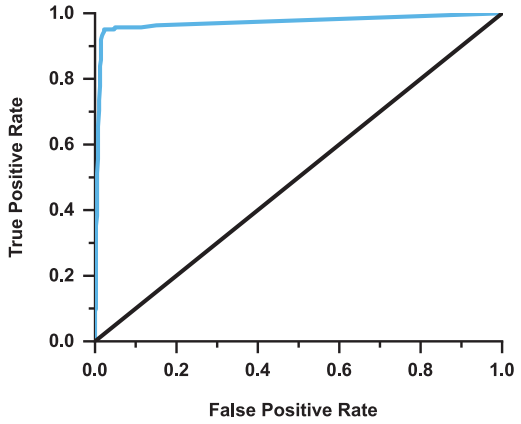

Figure 4. Receiver operator characteristic (ROC) curve illustrating the true positive rate (TPR) and false positive rate (FPR) of the bovine respiratory disease (BRD) loop-mediated isothermal amplification (LAMP) assay with multi-isolate data presented in Figure 2.

Table 4. Performance Characteristics of All Designed Primer Sets for Screening[a]

Primer Set	Response Time (min)	Response Time Spread (min)	Max Intensity (RFU)	Max Intensity Spread (RFU)	Total False Positives	Total Score
kmt1.1	13.5	0.6	19867.7	3140.6	4	0
kmt1.2	**12.3**	**0.5**	**23011.1**	**2308.1**	**0**	**91.4**
kmt1.3	15.8	0.5	18443.5	3842.2	0	85.1
ompP1.1	18.3	0.5	24363.8	3058.9	4	77.8
ompP1.2	**18**	**0**	**26217**	**2191.9**	**2**	**86.9**
ompP1.3	32.5	7	23301.9	2170.7	1	49.6
omp16.1	**18.8**	**0.5**	**21989.6**	**2149.6**	**0**	**86.8**
omp16.2	22.3	0.5	25324	2847	2	81
omp16.3	43	1.6	16808.9	1152.8	0	64.6
rsmL.1	26.3	2.2	27059.9	382.5	7	62.2
rsmL.2	**18.3**	**0.5**	**25720.1**	**4300.4**	**1**	**84.9**
rsmL.3	21.3	1.5	30923.3	5814.4	1	80.6
rsmC.1	19.3	1.5	30716.5	6274.2	10	58.9
rsmC.2	31.5	7.3	14235.6	6248.4	0	42.4
rsmC.3	**30.5**	**1**	**19127.2**	**1816.2**	**0**	**75.8**
lktA.1	25.3	1.3	18785.5	2121	10	52.7
lktA.2	20	0.8	24106.6	2765.7	7	0
lktA.3	**19**	**0**	**18906.3**	**1479.9**	**12**	**57.5**
lolA.1	22	1.4	22003.7	1838.9	0	81.3
lolA.2	**16**	**0**	**25501.2**	**1824.5**	**0**	**93**
lolA.3	13.8	1	24870.2	1616	2	85.4
lolB.1	35.5	0.6	23504.3	802	11	50.5
lolB.2	28	1.2	23133.7	1060.1	0	80
lolB.3	**16.5**	**0.6**	**22503**	**1461.9**	**0**	**88.9**
lppB.1	19.8	0.5	19569.2	1501.7	2	80.4
lppB.2	43.8	5.1	25406.9	11254.2	2	40.7
lppB.3	**23**	**0**	**22834.9**	**1485.6**	**0**	**87.3**

[a]Highlighted rows symbolize primer sets with optimal reaction features that were chosen for further assay development.

was an associated increase in the response time of all primer sets (Figures S3–S5). In general, the overall LoD of the LAMP reactions is worse than what is reported for PCR.[17] However, the difference between the maximum reported PCR LoD (250 copies/reaction) and the LAMP LoD of water samples indicates that conducting a more detailed LoD study between 10^2 and 10^3 copies/reaction may reveal a closer agreement between the limits of detection by using PCR or LAMP. Because we diluted the gDNA by factors of 10 when conducting the LoD study, it was a relatively coarse measurement and, thus, the performance of different primers seems quite similar. In the future, a finer study (for example, by using a 2× dilution factor) could highlight differences in the LoD for different primer sets. In addition, measuring the same gDNA sample by a separate technique such as droplet digital PCR could help confirm the accuracy of the measured LoD for LAMP.

Cross-Reactivity of LAMP Assays across Multiple Sample Isolates. We tested the specificity of our optimal primer sets by comparing the amplification results of LAMP primers with purified off-target DNA. On the basis of the multi-isolate data as shown in Figure 2, most of the primer sets amplify the target pathogenic DNA and do not amplify off-target pathogenic DNA. By generating ROC curves (Figure 3) for each pathogen based on the multi-isolate data, we determined the diagnostic sensitivities and specificities for each pathogen assay (Table 3). For the overall BRD LAMP assay, we used ROC analysis on the curve presented in Figure 4 and determined that the primers had 97% accuracy (95% sensitivity and 98% specificity) when using a fluorescence threshold of 28% of the maximum reported intensity.

Using different isolates of the same species helps check for cross-reactivity in case there were strain-specific genetic differences that could influence reaction performance. Because most isolates show consistent amplification results with their own species, our LAMP primer sets are functioning as expected. One exception was *H. somni* isolate 7896, which was not amplified reliably with any of our LAMP primers. Further sequencing and genome annotation using Rapid Annotation using Subsystem Technology (RAST) revealed that this isolate was putatively identified as *Staphylococcus hominis*, which has no significant similarity with *H. somni* and whose genomic DNA would not be expected to be amplified with our *H. somni* primer sets. It is likely that when this isolate was handled, it might have been mislabeled prior to collection from the ADDL or contaminated during culturing for DNA isolation.

Diagnostic abilities of BRD LAMP were either comparable to (sensitivity) or better than (specificity) reported qPCR values.[17,18] However, samples used for qPCR assays were derived from collected animal samples and subjected to DNA extraction procedures prior to use. Sample processing can cause variability in the total DNA load of on-target and off-target gDNA per sample used, which may contribute to poorer diagnostic performance. LAMP samples, in contrast, represented ideal sample conditions by being concentration-controlled additions of target gDNA in nuclease-free water, which would allow for optimal performance of the LAMP assay.

Evaluation of Primer Performance through Quantitative LAMP (qLAMP). We selected three genes for targeting each bacterial target. While some of these genes were published as PCR targets,[19] others were discovered by

https://dx.doi.org/10.1021/acsagscitech.0c00072
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Table 5. Screen-Selected Primer Set Sequences Used in Assay Development

primer set	sequence (5′ to 3′)
kmt1.2 F3	GAATCAAGCGGTCACAG
kmt1.2 B3	CACTCACAACGAGCCATA
kmt1.2 FIP	AGAGCAGTAATGTCAGCACAATATTAAAGACAGCAATTTCGAGCA
kmt1.2 BIP	CGCTATTTACCCAGTGGGGCGCCATTTCCCATTTCAAGTG
kmt1.2 LF	CGTAAAGCCCCACCATTGTT
kmt1.2 LB	ACCGATTGCCGCGAAATTGAGT
omp1.2 F3	GCAATTTATGTGGACCCAAAT
omp1.2 B3	AATCGGTTTTACCGCCTA
omp1.2 FIP	TCGGAACTAACGCATTCGGCTTAACTTCACCAATGCCAGG
omp1.2 BIP	ATCCAATTAACGAAAAATTCGCTGTGCATATTTGTCATCAAACTCGG
omp1.2 LF	CAATATTTTTATAGGCGAA
omp1.2 LB	GGCGGTGGATTGAATGTCAAC
omp16.1 F3	GGCGGTTATTCAGTACAAGA
omp16.1 B3	CATCTGCACGACGTTGAC
omp16.1 FIP	CGCATGTGCATCTAAAATTTGTACAGTTATAATACCGTGTATTTCGGC
omp16.1 BIP	AATGCAACACCTGCAACGAACTAATGCGATGTTATATTCTGGT
omp16.1 LF	CGATATTGTATTTATCGA
omp16.1 LB	CGTTGTTGAAGGTAACACCGA
lktA.3 F3	GTAACGACGGCAATGACC
lktA.3 B3	ATCTTTTAAGTTCGAATCAGAGA
lktA.3 FIP	TTGCCTTTACCGCCATCGATAAAGTAAAGGCGATGATATTCTCG
lktA.3 BIP	GGTGGCAAGGGCGATGATATCATTGCCGTCAGAATCGG
lktA.3 LF	TCATCACCATTTCCACCA
lktA.3 LB	TCGTTCACCGTAAAGGCGAT
rsmI.2 F3	CGAAGACACTCGCCACAG
rsmI.2 B3	AACTTTTACCCCGGCTTGG
rsmI.2 FIP	AACGACCGCTTTCTGCTGTTCATTATTGCTGAGCCACTACGG
rsmI.2 BIP	TGCGTTAATTTCCGATGCCGGACGGCAATGACGGACAAGA
rsmI.2 LF	GTGCAAGGCGAAAAACGGTTTTTTA
rsmI.2 LB	GCCACTGATTAGCGACCCG
rsmC.3 F3	CGGCAGACGTACTTTGGC
rsmC.3 B3	ATGCGTTTGCGACAATTCG
rsmC.3 FIP	GTGGAATGGTGGGTTGGAGACA-AGAGGGGGAAGTGGTAGC
rsmC.3 BIP	ACGGGGTCGATACCGCCTAC-GCTCACCGCCTTTGGTTA
rsmC.3 LF	CCGTTCATTAATGTGAGAGAACACA
rsmC.3 LB	TGGAGGAGTTGATTTTCCAAGCT
lppB.3 F3	AGCACAAAAAATACTGAGCA
lppB.3 B3	AGAGAAGGAGATTATTTGGAATG
lppB.3 FIP	TGTTGCCCATACTTCTAAGGTTAAATTTTGTAGCCTCAGTTTTCAAGC
lppB.3 BIP	ACCGAATAAACAAAGCTATCCGATTTTTGCTGATTTTGCTAATGCGG
lppB.3 LF	TTTCTCTGCTTCATAACC
lppB.3 LB	CGCACTTTCTTTGATAACTCTCGT
lolA.2 F3	AGTAATGTAACTTGGGCAAAT
lolA.2 B3	GCAATAATTTGACTTTCTTGAGG
lolA.2 FIP	CACTTGTTGTGTATAGTCAGCACTTCGGTTAATGAGTTACAAAATCG
lolA.2 BIP	ATGCACAGGGAAAAAAAATACAGCTGTTTCATTGTCCATACGAAAT
lolA.2 LF	ACACATCAATTTTATTTAA
lolA.2 LB	GGAAAAATACAACTCAAACGT
lolB.3 F3	GCTACGTGAAATGATTGGTATC
lolB.3 B3	CTTTTCAGAAGAATATCTTTGGGTA
lolB.3 FIP	GCCGACCTGATAATCTGAATTTTCATATTCCATTACAACAAATAGGGAAC
lolB.3 BIP	GCAAGCTTTACTTATTCAGTTGAGGATGCTTTGATCTGTTCGATAG
lolB.3 LF	CTGGTTGACCTTTTAGCC
lolB.3 LB	GAAGTTTGGAGTGCTGAC

comparing BLAST available genomes as explained in Materials and Methods (Table 1). We determined which LAMP primer sets are optimal for our target genes by first designing multiple primer sets per gene and then characterizing their performance. Initial screening of primer sets was carried out to identify sets that could amplify genomic DNA from a target pathogen, while maintaining little or no amplification on other pathogens or negative samples. All designed primers (Table S2) were run with genomic DNA in water to test for primer dimerization (early amplification of negative controls) or cross-reactivity

https://dx.doi.org/10.1021/acsagscitech.0c00072
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with off-target DNA. Moreover, primer sets with faster reaction speeds and more consistent amplification trends (smaller standard deviation) were given higher priority for selection. The results of the screening (Table 4 and Figures S6−S8) indicate that the following primer sets were considered optimal (see Table 5 for sequences): (i) kmt1.2, ompP1.2, and omp16.1 for *P. multocida*, (ii) rsmC.3, rsmL.2, and lktA.3 for *M. haemolytica*, and (iii) lolA.2, lolB.3, and lppB.3 for *H. somni*. The optimal primer sets were used for the LoD, multi-isolate, and cross-reactivity studies described here.

In this work, we developed a LAMP assay that can (i) specifically detect the presence of BRD-causing bacteria (*P. multocida*, *M. haemolytica*, and *H. somni*) in <45 min, (ii) detect pathogen DNA in both simple water samples and unprocessed bovine nasal samples, and (iii) potentially translate to field use due to its ease of incubation and amenability to more visual forms of detection.

A major limitation of LAMP as a mainstream assay for pathogen screening is the occurrence of false positives due to either poor reagent handling or carryover contamination from previous experiments. However, this concern can be minimized by employing multiple spaces for reaction preparation, pre-aliquoting required reagents to reduce contamination losses, and adding increased concentrations of UDG and UTP to degrade leftover amplicons in incubation environments as we have done here.[20]

Applications of this assay can be extended further by the following four steps: (i) expanding the list of pathogens to other bacteria (e.g., *Mycoplasma bovis*), viruses, and fungi associated with BRD, (ii) selecting genes associated with antibiotic resistance as LAMP targets to allow for timely diagnosis of drug-resistant strains prior to outbreaks, (iii) coupling reactions with pH- or magnesium-based indicators to allow for colorimetric inspection of assay results,[21−23] and (iv) converting the assay into a format more amenable for resource-limited, field-use settings to bypass sample shipment and lab processing altogether.

Developing a colorimetric assay is part of our ongoing work, and we expect it to avoid the need for lab-based equipment. With further development, we expect this assay to be used on cattle that display symptoms of BRD, i.e., depression, loss of appetite, changes in respiration, and elevated temperature. After the sick cattle have been removed from their pen, a nasal swab could be collected and used with the assay described here to determine which of the three important BRD pathogens (*P. multocida*, *M. haemolytica*, and *H. somni*) are present at elevated levels, and a treatment plan could be determined accordingly.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsagscitech.0c00072.

List of all generated LAMP primer sets for work, gel confirming the presence of *P. multocida* in base complex samples, Tt values of *P. multocida* primer sets from cross-reactivity data, amplification curves of LoD studies, amplification curves used in initial primer performance evaluation, and the MATLAB script used to generate metric scores for primer performance evaluation (PDF)

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Funding

This work is supported by the Agriculture and Food Research Initiative Competitive Grants Program Award 2020-68014-31302 from the U.S. Department of Agriculture National Institute of Food and Agriculture. This work is also supported by Purdue University's Colleges of Agriculture and Engineering Collaborative Projects Program 2018, the College of Agriculture and Wabash Heartland Innovation Network Graduate Student Support program, an Agricultural Science and Extension for Economic Development (AgSEED) grant, and the Disease Diagnostics INventors Challenge (created by the Purdue Institute of Inflammation, Immunology and Infectious Disease in partnership with the Department of Comparative Pathobiology, which contributed the funds to realize the project, the Indiana Clinical and Translational Sciences Institute, and the Indiana Consortium for Analytical Science and Engineering).

Notes

The authors declare the following competing financial interest(s): M.S.V. has interests in Krishi LLC, a company interested in licensing the technology developed here. The work performed here was not funded by Krishi LLC.

ABBREVIATIONS USED

LoD, limit of detection; ADDL, Animal Disease Diagnostic Laboratory; BHI, brain−heart infusion; BLAST, Basic Local Alignment Search Tool; DNA, deoxyribonucleic acid; dNTPs, deoxyribonucleotide triphosphate; FPR, false positive rate; gDNA, genomic DNA; NCBI, National Center for Biotechnology Information; PACUC, Purdue Animal Care and Use Committee; RAST, Rapid Annotation using Subsystem Technology; ROC, receiver operator characteristic; RPA, recombinase polymerase amplification; qPCR, real-time polymerase chain reaction; TPR, true positive rate; TSB, tryptic soy broth; BRD, bovine respiratory disease complex; PCR, polymerase chain reaction; LAMP, loop-mediated isothermal amplification; UDG, uracil DNA glycosylase; UTP, uracil triphosphate

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ACS Agric. Sci. Technol. XXXX, XXX, XXX−XXX