

# Specific Detection of *Klebsiella variicola* and *K. oxytoca* by Loop-Mediated Isothermal Amplification

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## Abstract

*Klebsiella* spp. are opportunistic pathogens with clinical, veterinary and plant-associated isolates. A previous study showed that bacterial ooze from wetwood of severely declined ironwood trees in Guam contained *Ralstonia solanacearum*, *Klebsiella variicola* and *K. oxytoca*. In this study, Loop-Mediated Isothermal Amplification (LAMP) detected *K. variicola* and *K. oxytoca* specifically, using unique primer sets designed individually for each organism. Each LAMP detected its target specifically, while showing negative results for non-target bacteria and negative controls. LAMP detected *Klebsiella* in inoculated-ironwood stem tissues and bacterial ooze. Due to the presence of plant inhibitors, different sampling protocols were tested. Soaking plant tissue samples to allow diffusion of bacteria into solution, followed by boiling, provided optimum detection of *Klebsiella* directly from plant samples. False negatives obtained when using crushed plant samples were eliminated by including an enrichment step, involving plating and 12-h incubation. DGGE (denaturing gradient gel electrophoresis) and a colony-blot immunoassay using a *Klebsiella*-specific antibody also detected *Klebsiella* in inoculated ironwood. DGGE bands and antibody cross-reactions from closely related enterobacters showed the potential for false positive results. The nature of LAMP makes it ideal for point-of-care testing, and when combined with the specificity of the LAMP primers developed in this study, demonstrates its potential as a routine field test for *Klebsiella* in ironwood in Guam, as well as clinical and veterinary diagnosis of *Klebsiella* infection. Additionally, the regions targeted for detection in this study have application across all forms of molecular-based diagnostics.

**Keywords:** *Klebsiella*; Detection; Loop-mediated amplification; *Casuarina equisetifolia*; ironwood; Decline

## Introduction

Ironwood (*Casuarina equisetifolia*) is a common forest tree in the Pacific islands and is widely propagated in Australia, India and China for firewood, wood for construction, land reclamation and windbreaks. Ironwood is salt tolerant and grows on nutrient-poor sandy soils common on coastlines [1]. For the past twelve years, ironwood trees on Guam have been undergoing a slow decline and thousands have died throughout the island [2-4]. Ironwood decline is associated with thinning foliage, dieback of branches, and wetwood formation. Various causal agents, including the root and butt rot fungus *Ganoderma* spp., termites, tree-care practices, and most recently bacteria, have been implicated [5]. However, the disease etiology has not been clearly established [2,4,6,7]. A disease showing similar symptoms was first reported in Mauritius [8], and later in India [9]. The disease symptoms include defoliation, yellowing, wilt, dieback and finally death of the trees.

In a 2011 survey of ironwood decline in Guam, involvement of *Ralstonia solanacearum* (*Rs*), a bacterial pathogen that causes wilt in a large number of plant hosts including ironwood, was suspected when ooze from diseased trees produced positive reactions with *Rs*-specific immunostrips (Agdia, Inc.). Following the initial observation by Putnam in 2011 [10], numerous samples from symptomatic plants were sent to Hawai'i for isolation and identification. However, *Rs* could not be cultured, resulting in an additional survey in Guam in 2012. Three complimentary approaches were used to isolate and identify the pathogen: growth on modified *Rs*-semiselective medium (mSMSA), *Rs*-specific immunostrip assays, and confirmation with a *Rs*-specific loop-mediated isothermal amplification (LAMP) reaction [11,12]. The

identity of isolates was determined in Hawai'i and the two predominant types of bacteria associated with ironwood decline were characterized [13,14]. Bacteriological tests, along with multiplex PCR, BOX-PCR, and phylogenetic analysis of the *dnaA* RIF marker [15], were performed to further characterize and evaluate the genetic diversity among the *Rs* isolates [13,14]. Non-*Rs* isolates were presumptively identified as *Klebsiella* spp. using bacteriological tests [13,14,16,17]. Most strains showed closest similarity to *K. variicola* following sequence analysis of the 16S rRNA gene, while one showed closest match to *K. oxytoca*. Strains identified as *Rs* were capable of wilting tomato seedlings within 7-10 days and ironwood seedlings within 10-30 days. *Klebsiella* strains did not produce symptoms in inoculated plants, but were re-isolated from stem tissues as far as 10 cm above the inoculation site, indicating possible colonization of the plant. Results indicated, for the first time, that *Rs* is involved in the decline of ironwood in Guam and *Klebsiella* spp. are associated with wetwood, a phenomenon associated with various bacteria and reported in other forest ecosystems [18,19].

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Received February 25, 2015; Accepted May 20, 2015; Published June 25, 2015

**Citation:** Yasuhara-Bell J, Ayin C, Hatada A, Yoo Y, Schlub RL, et al. (2015) Specific Detection of *Klebsiella variicola* and *K. oxytoca* by Loop-Mediated Isothermal Amplification. J Plant Pathol Microb 6: 271. doi:10.4172/2157-7471.1000271

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Ironwood decline has not yet been observed in Hawai'i, or even Saipan, which is much closer to Guam, even though *Rs* is present throughout the Pacific. Molecular and immunodiagnostic tools for rapid pathogen identification are needed to obtain relevant data in future field studies to better understand ironwood decline and its potential to spread to other islands of the Pacific. Immunostrips (Agdia, Inc.) and LAMP technology using a hand-held SMART-DART device [11,12] are available for *Rs* detection, but similar tests are needed to determine the possible role of *Klebsiella* spp. in ironwood decline. Once developed and validated, field-ready diagnostic tools can be used to determine the association of these two bacteria within naturally infected symptomatic plants, and lack of association with healthy ones. These assays could also be used to screen nursery trees for latent and active infections, thereby providing tools to produce healthy nursery stocks for the replenishment of dead ironwood trees and reforestation in Guam. This study compares several diagnostic approaches, including a LAMP assay, for detecting *Klebsiella* spp. in ironwood, as well as mixed cultures with *Ralstonia solanacearum* and other bacterial genera.

## Materials and Methods

### Bacterial strains and culture conditions

The strains used in this study (Table 1) were from the Pacific Bacterial Collection at the University of Hawai'i at Mānoa and included three *Klebsiella variicola* strains (A6126, A6127, and A6128), one *K. oxytoca* strain (A6125) and two *Ralstonia solanacearum* strains (A6123 and A6124), which were all isolated from ironwood in Guam, one *K. pneumoniae* strain (A6133, aka ATCC 13883), as well as *Agrobacterium tumefaciens* (A2961, aka C58), *Enterobacter aerogenes* (A3131, aka ATCC 13048), *E. cloacae* (A5149), *Escherichia coli* (A6091, aka LJH 1947), *Pseudomonas fluorescens* (A3275, aka A811-1), and *Ralstonia solanacearum* (GMI 1000). Bacteria were removed from storage, plated onto TZC medium (17 g/L agar, 10 g/L peptone, 5 g/L glucose and 0.001% 2,3,5-triphenyl-tetrazolium chloride (TZC)) and then incubated at 26°C (±2°C).

### Study design

DNA from *Klebsiella* spp. and *Ralstonia solanacearum* strains were tested individually, as well as in mixtures of two different genera or species. Individual and mixed DNA were further tested in the presence of plant tissue. Molecular tests of DNA included PCR followed by DGGE (denaturing gradient gel electrophoresis) analysis and LAMP. DNA from non-*Klebsiella* and non-*Ralstonia* bacteria were also tested with LAMP to determine specificity.

Cultures of *Klebsiella* spp. and *Ralstonia solanacearum* strains were used individually, as well as in mixtures of two different genera or species, and tested with a colony-blot assay, in the absence and presence of plant tissue. Cultures were also inoculated into plants individually and as mixtures. Samples were taken from plants and tested with LAMP, DGGE, and colony-blot assay.

### Plant inoculation

Plant inoculations were performed according to a previously established protocol [13,14]. Briefly, bacterial suspensions at 10<sup>8</sup> CFU/ml were prepared from 48 hr cultures of test strains from TZC plates. Seeds of *C. equisetifolia* trees were collected from the University of Hawai'i at Mānoa campus, germinated on damp filter paper, placed in the dark and then transplanted to community pots. Established seedlings were transplanted to 6.7 cm pots containing Sunshine Mix #4 (Sun Gro Horticulture, Agawam, MA). Roots of 15-week-old seedlings

were wounded by drawing a sterile scalpel through the soil at four sides of the pot in the root zone. Then, 10 ml of the bacterial suspension were pipetted into the soil after the wounding process. Older ironwood seedlings, ~17 weeks old, were inoculated with 50 ml of bacterial suspension in the same manner. Two plants were co-inoculated with *Ralstonia solanacearum* and *Klebsiella variicola*, but one was an older, 10-month old ironwood (Plant 1) that was used to try to produce bacterial ooze. The ironwood seedlings were then grown under the same conditions as described previously [14].

### Plant sampling

Plant samplings were performed according to a previously established protocol [14]. Briefly, stem sections of plants were surface sterilized in 10% Clorox (final concentration: 0.8% sodium hypochlorite) for 30 seconds and triple-rinsed in sterile de-ionized water. Stem sections were then macerated in ~200 µl of sterile de-ionized water and the subsequent suspension was streaked onto TZC. Following a 24 h enrichment phase on TZC, the identity of the re-isolated bacteria was presumptively determined by appearance, and then confirmed using the various tests described hereafter. Tests were also performed directly on the extracted plant samples [15-19]. Bacterial ooze from plant samples was also collected by soaking stem sections in water to allow bacteria to diffuse from the plant material. For co-inoculated plants, five sample sections were taken from Plant 1, while eight were taken from a younger one (Plant 2), with sections being numbered sequentially away from the inoculum site. Tests were performed directly on bacterial ooze, as well as isolated colonies following a 12-h and 24-h enrichment.

### DNA extraction

A Chelex DNA extraction was performed on isolated bacteria, as well as plant samples. Briefly, 0.75-1.0 ml of 40% Chelex 100 resin (Bio-Rad, Hercules, CA) in 1X TE buffer (10 mM Tris HCl and 1 mM EDTA at pH 8) with 10% Triton X-100 (Sigma-Aldrich, St. Louis, MO) was added to each sample. Samples were mixed by pipeting vigorously and vortexing and then heated to 95°C for 10 minutes on a digital heat block. Samples were stored at 4°C. Various other DNA extraction protocols were used on plant samples to remove inhibitors [20-24], including the PowerPlant Pro DNA isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA).

### DGGE

DGGE primers (341F(-GC)/907RA) used in this study were reported previously to amplify the variable V3 region of the 16S rRNA gene [25-27]. PCR reactions for 341F(-GC)/907RA were performed in

Organism	Strain	Other ID	Hcp 1 LAMP	UGH LAMP
<i>Agrobacterium tumefaciens</i>	A2961	C58	-	-
<i>Enterobacter aerogenes</i>	A3133	ATCC 13048	-	-
<i>Enterobacter cloacae</i>	A5149	B193	-	-
<i>Escherichia coli</i>	A6091	LJH 1947	-	-
<i>Klebsiella oxytoca</i>	A6125	S-3	-	+
<i>Klebsiella pneumoniae</i>	A6133	ATCC 13883	-	-
<i>Klebsiella variicola</i>	A6126	S-4	+	-
<i>Klebsiella variicola</i>	A6127	S-8	+	-
<i>Klebsiella variicola</i>	A6128	S-19	+	-
<i>Pseudomonas fluorescens</i>	A3275	A811-1	-	-
<i>Ralstonia solanacearum</i>	A6123	S-25	-	-
<i>Ralstonia solanacearum</i>	A6124	S-26	-	-
<i>Ralstonia solanacearum</i>	A3292	GMI-1000	-	-

**Table 1:** Bacterial strains used in this study and corresponding LAMP results.

a 25 µl reaction volume containing 1 µl bacterial DNA and 24 µl PCR reaction master mix [1 µl of each primer (10 µM), 0.13 µl GoTaq DNA polymerase (5 U/ µl) (Promega, Madison, WI), 5 µl 5X GoTaq Reaction Buffer (Promega, Madison, WI), 0.5 µl dNTP mix (10 mM) (Promega, Madison, WI) and 16.37 µl DNase/RNase free water]. PCR reaction conditions were as follows: an initial denaturing at 94°C for 5 min, followed by 25 cycles of denaturing at 94°C for 30 s, annealing at 57°C for 1.5 min, and elongation at 72°C for 1 min, with a final elongation at 72°C for 10 min. PCR products were resolved using DGGE. PCR primer sequences are listed in (Table 2).

DGGE analysis was performed with a DGGE-2000 system (C.B.S Scientific Co.), using a 0.75 mm-thick 6% polyacrylamide gel (ratio of acrylamide to bis-acrylamide, 37.5:1) with a linear denaturing-agent gradient of 35-65% (100% denaturant agent was defined as 7 M urea with 40% formamide) that was submerged in 0.5X TAE buffer (40 mM Tris, 40 mM acetic acid, 1 mM EDTA; pH 7.4) at 60°C. PCR samples were mixed with 6 µl of dye solution [0.1% bromphenol blue (w/v), 70% glycerol (v/v)] and applied to the gels. Electrophoresis conditions were 5 h 30 min at 75 V. Gels were stained for 30 min in 1X TAE buffer with ethidium bromide and visualized using the Foto/Analyst Express System (Fotodyne Inc., Hartland, WI). Products were excised from gels and resuspended in sterile water [28]. Products were reamplified using 341F/907RA primers (Table 2), under the same reaction conditions, and then sequenced for identification. Sequences were queried in the National Center for Biotechnology Information (NCBI) database using the Basic Alignment Search Tool (BLAST) [29].

### DNA sequencing

PCR products were cleaned for sequencing using ExoSAP-IT (Affymetrix, Santa Clara, CA), according to the manufacturer's instructions. Cleaned PCR products were sequenced at the Greenwood Molecular Biology Facility at the University of Hawai'i sequencing facility, using each forward and reverse primer, according to specifications.

### Colony-blot immunoassay

The colony blot immunoassay was performed according to methods established previously [30,31], with modifications. Briefly, 1 µl of bacterial suspension (OD<sub>A600</sub>=0.1), or plant extract, were spotted onto 0.45 µm nitrocellulose membranes (NitroBind, Osmonics, Inc., Minnetonka, MN) and allowed to dry completely. Dried nitrocellulose membranes were blocked in 5% PBS-milk for 1 h and then boiled in PBS for 10 min to eliminate endogenous enzymatic activity. Membranes were then washed three to five times using 0.05% PBS-Tween 20. Membranes were subsequently incubated with *Klebsiella* species antibody (73/28) (ThermoFisher Scientific, Waltham, MA) at a 1:2000 dilution for 30 min, followed by washing three times for five minutes each with 0.5% PBS-Tween 20. Membranes were then incubated with goat anti-mouse antibody conjugated to alkaline phosphatase (1:2000) (Southern Biotech, Birmingham, AL) for 30 min and washed three times for five minutes with 0.5% PBS-Tween 20. Membranes were developed with 1-Step NBT/BCIP (ThermoFisher Scientific, Waltham, MA) for 15-30 min, rinsed with distilled water, and air-dried. Colony immunoblots were analyzed under a dissecting microscope.

### LAMP

LAMP primers developed in this study are shown in Table 2. Individual LAMP reactions were performed in triplicate and contained 5 µl sample, 5 µl primer master mix [F3 (0.2 µM), B3 (0.2 µM), FIP (1.6 µM), BIP (1.6 µM), Loop (0.8 µM), Loop Probe (0.08 µM), and Quencher probe (0.16 µM)] and 15 µl ISO-001nd Isothermal Mastermix (OptiGene, West Sussex, UK). Hcp1 and UGH primers were multiplexed to produce a reaction to detect both *Klebsiella variicola* and *K. oxytoca*, requiring 0.4 µM Quencher probe. Negative controls used 5 µl ddH<sub>2</sub>O and/or pathogen-free plant samples. LAMP reactions were run and analyzed using the iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA) and a hand-held real-time assessment device (SMART-DART) (DiaGenetix Inc., Honolulu,

Oligonucleotide Primer Sequence (5'-3')	Source/Reference	
LAMP		
Hcp1-F3	CCCCATACCTTTACAAGGCC	This Study
Hcp1-B3	CGTGGATCCTCCAGGTGATT	This Study
Hcp1-FIP	GCTGAGTTTCACTCTTGAATACCAGCATTATGAACTTCGAACGT	This Study
Hcp1-BIP	ACCGGGGTTAACTGTGGTGTACTGCAGGCTAACGCTTTCAAC	This Study
Hcp1-Loop	TGCAAACCTTTCCTCGAATGACA	This Study
Hcp1-Loop Probe	/56-FAM/ ACGCTGAGGACCCGGATGCGAATGCGGATGCGGATGCCGATGCCAACTTTCTCGAATGACA	This Study
UGH-F3	TGCCAGCAGACATTGACG	This Study
UGH-B3	CCGACCACTACGAACGGT	This Study
UGH-FIP	CGATTGCGATCTGCGGCCTGCGCCAGTTTCTGGTAACCG	This Study
UGH-BIP	TCTGCAGCCAGCAGCAGATCGCCTGCGAGTATTTCTTCCG	This Study
UGH-Loop	GCGCATAAGCACTTTCCGG	This Study
UGH-Loop Probe	/56-FAM/ ACGCTGAGGACCCGGATGCGAATGCGGATGCGGATGCCGATTTGCGCATAAGCACTTTCCGG	This Study
Quencher probe	TCGGCATCCGCATCCGCATTCGCATCCGGTCTCAGCGT/3BHQ_1/	Kubota et al., [11]
PCR		
<i>gyrA</i> -A	CGCGTACTATACGCCATGAACGTA	Brisse and Verhoef, [42]
<i>gyrA</i> -C	ACCGTTGATCACTTCGGTCAGG	Brisse and Verhoef, [42]
DGGE		
341F	CCTACGGGAGGCAGCAG	Muyzer et al., [25]
341F(-GC)	(CGCCC GCCGCCCCGCGCCCGTCCCGCCGCCCGCCCGC)-CCTACGGGAGGCAGCAG	Muyzer et al., [25]; Meyer and Kuever, 2008
907RA	CCGTCAATTCATTTGAGTTT	Muyzer et al., [26]; Ishii and Fukui, [27]

\*/56-FAM/: 5' 6- carboxyfluorescein; /3BHQ\_1/: 3' Black Hole Quencher 1

Table 2: Oligonucleotide primers used in this study\*.

HI), under the following conditions: 65°C for 30 min, with fluorescence readings being taken at 30-s or 1-min intervals. One drop of mineral oil was added to the tops of each sample when using the SMART-DART to prevent evaporation.

### Sensitivity assay

Bacterial DNA was isolated using the Wizard Genome DNA Purification Kit (Promega, Madison, WI). DNA was quantified using the NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA) and diluted to a starting concentration of 1 ng/μl, with subsequent 10-fold serial dilutions made to reach a final concentration of 1 fg/μl. LAMP reactions were performed in triplicate, using ddH<sub>2</sub>O as the negative control.

## Results

### DGGE

DGGE analyses were performed to determine its effectiveness in identifying *Ralstonia* and *Klebsiella* from inoculated ironwood. Samples were analyzed alongside positive controls (Figure 1). Positive controls consisting of purified DNA samples of *Ralstonia*, *Klebsiella*, and mixes of the two former (Figure 1, Lanes 1-3), show a characteristic *Ralstonia* and *Klebsiella* band (Figure 1, Lane 1 and Lane 2, respectively), with both bands appearing in the mixed sample (Figure 1, Lane 3). A mixed sample containing DNA from purified *Ralstonia* and *Klebsiella*, as well as DNA from *Enterobacter aerogenes*, *E. cloacae* and *Escherichia coli*, which are closely related to *Klebsiella* and found associated with ironwood, produced the characteristic *Ralstonia* and *Klebsiella* bands, however additional bands reflecting all of the additional bacteria contained within the sample were not present (Figure 1, Lane 4). Plants samples from ironwood inoculated with *Ralstonia*, *Klebsiella*, and both *Ralstonia* and *Klebsiella* produced the appropriate characteristic *Ralstonia* and *Klebsiella* bands (Figure 1, Lanes 5-7), with the *Klebsiella* band being faint in the plant sample with mixed infection (Figure 1, Lane 7). All characteristic bands were excised, re-amplified and sequenced. BLAST analysis of returned sequence data revealed that the characteristic *Ralstonia* and *Klebsiella* bands were indicative of each respective bacterium, thus demonstrating the utility of DGGE, and its potential ability to be used without sequencing by including appropriate controls. However, in the mixed sample, which contained other enteric bacteria (Figure 1, Lane 4), sequence data could not be obtained for the *Klebsiella* band. A strong band was produced for *Ralstonia solanacearum*, a weak band for *Klebsiella*, and no distinct bands for all other enteric bacteria. Similarities between bands of the enteric bacteria and *Klebsiella* could explain the lack of additional bands. Additionally, the *Klebsiella* band in the mixed plant sample was very faint (Lane 7). Inefficient recovery of DNA from the excised band is responsible for the inability to obtain sequence data.

### Colony-blot assay

A *Klebsiella*-specific antibody was tested for specificity using pure bacterial suspensions in a colony-blot immunoassay format (Figure 2a). Positive reactions were obtained with all three *Klebsiella* spp. tested and negative reactions with *R. solanacearum*, *P. fluorescens* and the water control. However, *Enterobacter aerogenes*, *E. coli*, and *Agrobacterium tumefaciens* gave cross-reactions with the *Klebsiella* antibody assay. The colony-blot assay detected *Klebsiella* in spiked plant samples (Figure 2b). Positive controls made with pure bacterial suspensions of *Klebsiella* were positive, while negative controls consisting of *Ralstonia*, only macerated plant tissue, and water, were all negative. Plant samples containing *Klebsiella variicola* and *K. oxytoca* showed

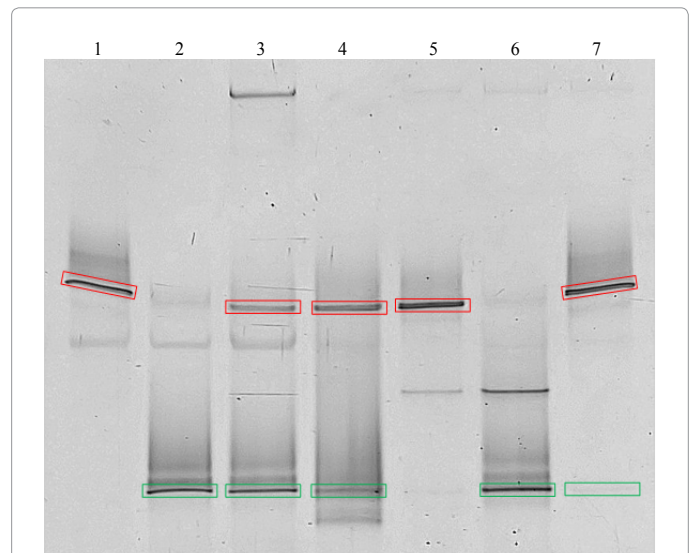
positive reactions, while plant samples containing *Ralstonia* did not, thus demonstrating potential for use in detecting *Klebsiella* from plant tissue samples.

### LAMP

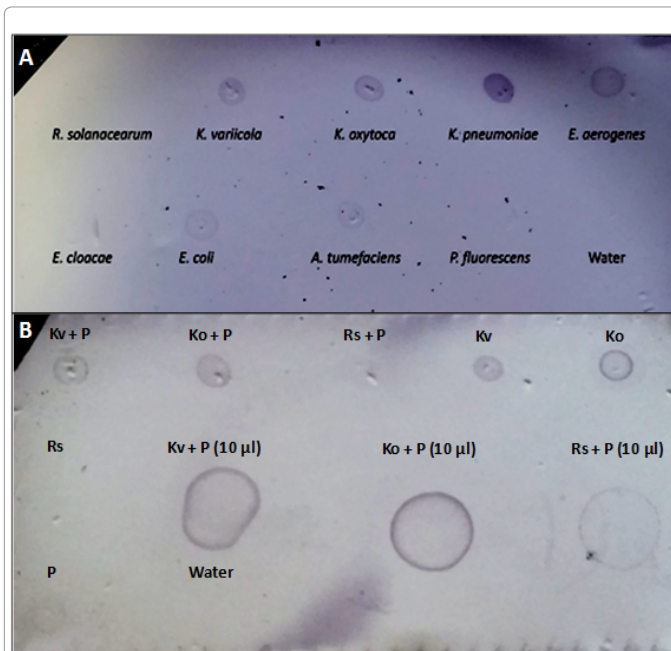
Two sets of LAMP primers were designed to specifically detect *K. oxytoca* and *K. variicola*. Both LAMP reactions were highly specific, only detecting their respective *Klebsiella* species and producing no reaction with any of the other bacteria tested in this study (Table 1), as well as negative controls. Positive LAMP reactions were recorded visually via turbidity, using the specified primers and a LAMP reaction master mix (dNTP mix (1.2 mM), 10X ThermoPol Reaction Buffer (New England Biolabs, Ipswich, MA) (2 mM), betaine (1 M), MgSO<sub>4</sub> (4 mM), *Bst* DNA polymerase (New England Biolabs, Ipswich, MA) (8 U) and ddH<sub>2</sub>O) that was described previously [32,33]. Under the current protocol, positive reactions were visible in real-time and commonly occurred within 10-20 minutes.

The sensitivity of the Hcp1-LAMP reaction was determined and DNA in the range of 5 ng to 5 pg showed positive reactions in approximately 10 minutes, while DNA in the range of 500 fg to 5 fg showed positive reactions in approximately 15 minutes. However, at the 5 fg range of DNA, positive reactions were not always obtained due to the low amount of DNA in the starting sample. The sensitivity of the UGH-LAMP reaction was less than that of the Hcp1-LAMP, with a detection limit of ~5 pg of DNA.

LAMP was performed on plant sample extracts following a 12-h and 24-h enrichment phase on solid media. All samples from known inoculated plants were positive by LAMP, while negative control plants were negative by LAMP. Non-*Klebsiella* bacteria that were



**Figure 1:** DGGE analysis using a linear denaturing-agent gradient of 35-65%. DNA from samples were amplified using DGGE-specific primers designed against the 16 rRNA gene. Lanes 1 and 2: positive controls using purified *Ralstonia* and *Klebsiella* DNA, respectively. Lane 3: additional positive control consisting of mixed purified *Ralstonia* and *Klebsiella* DNA. Lane 4: mixed sample consisting of mixed purified *Ralstonia* and *Klebsiella* DNA, as well as DNA from *Enterobacter aerogenes*, *E. cloacae* and *Escherichia coli*, which are closely related to *Klebsiella* and found associated with ironwood. Lane 5: *Ralstonia*-inoculated plant sample. Lane 6: *Klebsiella*-inoculated plant sample. Lane 7: plant sample inoculated with *Ralstonia* and *Klebsiella*. Red boxes indicate the characteristic *Ralstonia* band. Green boxes indicate the characteristic *Klebsiella* band. Characteristic bands were excised and sequenced from all lanes.



**Figure 2:** Colony-blot immunoassay using a *Klebsiella*-specific antibody. Nitrocellulose membranes were spotted with (A) 1 µl bacterial suspension (ODA600=0.1) or (B) spike plant samples and allowed to dry. A colony-blot immunoassay was performed on membranes using a *Klebsiella*-specific antibody. Secondary antibody was a goat anti-mouse antibody conjugated to alkaline phosphatase. Membranes were developed with 1-Step NBT/BCIP, which yields a purple color as a positive reaction. A faint spot is visible for the 10 µl *Ralstonia*-spiked plant sample; however that was attributed to background staining of the membrane by plant tissue itself, as seen by the faint spot for the non-spiked plant sample. Kv: *Klebsiella variicola*; Ko: *Klebsiella oxytoca*; Rs: *Ralstonia solanacearum*; P: plant extract.

present in the plant samples were tested separately and were negative with LAMP. In order to make a single test for both *K. variicola* and *K. oxytoca*, the LAMP primers were multiplexed into a single reaction. The multiplexed test was able to detect both *Klebsiella* spp.; however the sensitivity of the combined assay was compromised, as known to occur during multiplexing [34,35].

LAMP was further tested directly on plant sample extracts from two co-inoculated plants. Extracts were streaked onto plates and the first two sections of Plant 1 showed growth of *Klebsiella* and *Ralstonia*, while only *Ralstonia* grew from sections 3-5. In Plant 2, *Klebsiella* grew only from the first five sections. Initial testing using turbidity revealed that plant inhibitors did affect the LAMP reaction to a certain degree (data not shown). For real-time LAMP detection, extracts were processed, using various methods, and tests revealed no significant difference in results between direct boiling, 1:1 sample and TE buffer with boil, Chelex extraction, or GES buffer, using buffers 1 and 2 and sample at a 0.5:0.5:1 ratio. All *Klebsiella*-negative samples, including negative and *Ralstonia* controls, were all negative by LAMP. The LAMP reaction did not react with boiled samples from Plant 2 at sections 1, 3 and 4, TE-buffered samples from Plant 2 at section 2, and GES-buffered samples from Plant 1 at section 2. All other samples were positive by LAMP, including all Chelex-extracted samples. Included in the LAMP test was one *Klebsiella*-inoculated plant sample that had DNA extracted using the PowerPlant Pro DNA isolation kit, which is explicitly designed to extract DNA from plants and remove plant inhibitors. This sample was positive by LAMP, and reactions were the most robust; however, this kit is high in cost and therefore not practical for field use/studies. The

SMART-DART hand-held real-time fluorescence monitoring device performed equally to the in-lab real-time machine.

## Discussion

The genus *Klebsiella* is comprised of opportunistic pathogens that are frequently isolated from humans and animals [36]. In humans, *Klebsiella pneumoniae* and *K. oxytoca* are particularly involved with nosocomial infections [36,37], such as septicemia, pneumonia and urinary tract infection. In animals, *Klebsiella* spp. are mostly associated with sepsis, infections of the urinary and respiratory tracts, and mastitis, with *Klebsiella pneumoniae* and *K. oxytoca* being isolated frequently from domestic livestock [38-40]. These disease syndromes can cause serious economic consequences in some cattle herds [41]. The ecological habitats of *Klebsiella* include surface water, sewage, soils and plants, as well as mucosal surfaces of mammals [42]. Recently, a new species of *Klebsiella* was proposed, *K. variicola*, having clinical and plant-associated isolates [43].

In Guam, the association found between ironwood decline and *Ralstonia* led to the discovery of an association between *Klebsiella* and the same disease [11,12]. An association was also found between wetwood symptoms observed in infected trees and *Klebsiella* spp., which were determined to be *Klebsiella oxytoca* and *K. variicola* [13,14]. The current study, which focused on evaluation and design of diagnostic tools, will enable a more extensive survey of Guam ironwood trees to further investigate the association between *Klebsiella*- and *Ralstonia*-infected ironwood trees. DGGE, an immunoassay and a molecular-based assay (LAMP) were all tested for their ability to identify *Klebsiella* in ironwood.

DGGE is a method used to discern bacterial populations in mixed environmental samples. However, the complexity of DGGE makes it less desirable as a routine test for field studies. Additionally, results generally require DNA sequencing of bands following gel separation, which is not readily available to researchers in the field. Here, we attempted to circumvent the need for sequencing. Knowing what specific bacteria to look for and by running appropriate controls, we thought characteristic bands could be found that would be representative of *Ralstonia* and *Klebsiella*. While we were able to demonstrate this, the inability of DGGE to separate bands from closely related *Enterobacter* species contained in a mixed sample showed the possibility for false positive results. This fact, in combination with the laboriousness of DGGE, resulted in not recommending it as a possible test to survey Guam ironwood for *Klebsiella* infection.

Immunostrips (Agdia, Inc.) are available for detection of *Ralstonia*, but there are none for detection of *Klebsiella*. We explored the possibility of making an immunoassay that would be later commissioned into immunostrips for rapid detection of *Klebsiella*. We decided to test a commercial *Klebsiella* antibody for its potential as a rapid detection system, using a simple colony-blot test. As with most immunoassays, we observed cross-reactions with closely related bacteria. The *Klebsiella* antibody was made using *Klebsiella aerogenes*. *Klebsiella* species are constantly being reclassified into other genera and/or species. Interestingly enough, a very strong cross reaction was observed with *Enterobacter aerogenes*, even stronger than the interaction with *Klebsiella oxytoca* and *K. variicola*. Since this *Klebsiella* antibody reacted with a closely related *Enterobacter* sp. associated with ironwoods, this immunoassay is not recommended for routine field testing of *Klebsiella*-infected ironwood in Guam.

The potential for false positive results using the immunoassay and

the inability to clearly separate bands using DGGE led to the design of a specific molecular-based assay using loop-mediated amplification technology. The LAMP assay proved to be the most useful in this study. It was able to specifically detect *Klebsiella*, having no positive reactions with non-*Klebsiella* bacteria, and the two primer sets allowed differential detection of *Klebsiella oxytoca* and *K. variicola*. LAMP primers in this study were designed to amplify a hemolysin-coregulated protein (Hcp1) family type VI secretion system (T6SS) effector (*K. variicola* specific) and an unsaturated glucuronyl hydrolase (UGH) (*K. oxytoca* specific). These regions were found by whole-genome comparisons. BLAST searches of these genes resulted in matches only to each designated *Klebsiella* species, demonstrating that these primer sets should be highly specific for each *Klebsiella* species to which they were designed. LAMP tests using non-target bacteria confirmed the specificity of these genes as targets. Testing a broader panel of non-target bacteria would improve specificity claims; however, in silico data from BLAST searches combined with test results provided in this study, together, suggest a high specificity.

As with any molecular-based assay, plant inhibitors are always a concern. Once an effective assay is developed, sample processing remains the underlying factor. The LAMP assays presented here were affected by plant inhibitors to an extent. Plant samples produced some false negatives, but there were no false positive results and Chelex extraction was able to remove enough inhibitors to allow proper detection directly from woody tissues. Ultimately, to avoid any problems with plant inhibitors, it is recommended to simply enrich samples on solid media [17,44,45]. *Klebsiella* grows extremely fast and a simple 12-h incubation on media will eliminate all false results.

LAMP was chosen because it is the most widely researched isothermal nucleic acid method, which allows greater trouble-shooting during development [46]. LAMP is comparable to equivalent PCR-, immunoassay- or culture-based detection methods [47], with detection limits as low as five copies [48]. Moreover, its isothermal nature makes it suitable for field diagnostics.

Molecular methods such as PCR have been evaluated for rapid identification of *Klebsiella* in human clinical specimens [37,49]. However, *Klebsiella* species are difficult to identify and are often misclassified in clinical microbiology laboratories [16,50,51]. Phenotypic distinction between *K. pneumoniae* and *K. oxytoca* isolates based on existing biochemical tests are time consuming, laborious and not very reliable [52]. Current procedures, such as BIOLOG and API systems, often fail to differentiate between species of *Klebsiella* [50,53]. Differentiation of *Klebsiella* is a key component of clinical responses to infection. In Japan, in 2013, a patient with sepsis caused by *Klebsiella variicola* died due to misidentification as *Klebsiella pneumoniae* by a commonly used automated identification system [54]. The need for diagnostic tests that can properly identify *Klebsiella* at the species level has been nationally recognized, as the National Institute of Health and the National Institute of Allergy and Infectious Disease recently put out a request for proposal for an R01 grant that included *Klebsiella* as a target organism (Funding Opportunity Announcement Number RFA-AI-14-019).

The LAMP assays developed for *Klebsiella* have potential as clinical diagnostic tests because LAMP is less sensitive than PCR to inhibitory substances present in biological samples [55] such as serum [56], CSF [57], swabs [58], and heat-treated blood [59], saving time and money required for sample processing steps [60]. As a result, this technology has been used widely for molecular detection of several microorganisms, including clinical and plant-associated bacterial

[32,61-73], fungal [74-76], viral [47,77-84], and parasitic [57,59,85-88] pathogens, and is therefore suitable as a rapid field test.

Due to the isothermal nature of LAMP, it offers an easy and rapid assay for clinical medicine [89]. The LAMP reactions presented in this study not only specifically detect *Klebsiella*, but also distinguish *K. oxytoca* and *K. variicola*. Moreover, the target regions that these LAMP primers were designed to could become the new focus for developing *Klebsiella*-detection systems, having application to numerous molecular detection platforms. In conjunction with portable real-time fluorescence monitoring devices, such as the SMART-DART, these LAMP reactions could provide beneficial point-of-care diagnostics for clinical, veterinary and agricultural *Klebsiella* spp.

#### Acknowledgements

This work was supported in part by USDA National Institute for Food and Agriculture, Project HAW00987-H, administered by the College of Tropical Agriculture and Human Resources, University of Hawai'i at Mānoa. This work was also supported in part by WPDN-201303063-01 and by projects administered by the University of Guam: US Forest Service project 12-DG-11052021-236, USDA NIFA, RREA project 231395 and USDA NIFA, McIntire-Stennis project 1005476. The authors thank Daniel Jenkins and Ryo Kubota of DiaGenetix Inc. (Honolulu, HI) for providing the Isothermal Mastermix and SMART-DART.

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