

## ORIGINAL ARTICLE

# Rapid point-of-care diagnostics for the detection of *Chlamydia pecorum* in koalas (*Phascolarctos cinereus*) using loop-mediated isothermal amplification without nucleic acid purification

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## Abstract

Infectious disease, predominately chlamydiosis, contributes significantly to the decline in health of wild koala (*Phascolarctos cinereus*) populations in some regions of Australia. In this study, we describe the development and evaluation of a simple, sensitive, and specific loop-mediated isothermal amplification (LAMP) assay for the detection of *Chlamydia pecorum* in koalas as a point-of-care diagnostic tool that can be used in any wildlife hospital and in the field on specialized instrumentation. A set of primers targeting a 188-bp region of the *C. pecorum* genome was designed. 100% specificity of the LAMP assay was revealed by demonstrating no cross-reactivity with 33 nontarget pathogens, and complete correlation with qPCR results for 43 clinical swabs collected opportunistically from wildlife hospitals. In sensitivity evaluations, the technique successfully detected serial dilutions of extracted *C. pecorum* DNA with a detection limit of 44 IFU/ml.

## KEYWORDS

*Chlamydia pecorum*, diagnostics, koala, loop-mediated isothermal amplification, point-of-care

## 1 | INTRODUCTION

Spanning over 15 years, approximately 50% of koalas admitted to South East Queensland (SEQLD) wildlife hospitals have presented with chlamydiosis, frequently in combination with trauma and body wasting (Gonzalez-Astudillo, Allavena, McKinnon, Larkin, & Henning, 2017). The obligate intracellular bacteria *Chlamydia pecorum* is the most pathogenic and prevalent bacteria affecting koalas (Devereaux, Polkinghorne, Meijer, & Timms, 2003; Jackson, White, Giffard, & Timms, 1999; Marsh, Kollipara, Timms, & Polkinghorne, 2011) and infects the ocular, urinary, and genital mucosae with acute and/or persistent exposure to infection resulting in blindness, cystitis, and infertility (Blanshard & Bodley, 2008; Wan et al., 2011). Not all infections manifest in overt disease, and approximately half the koalas in SE Queensland appear to be healthy carriers of *C. pecorum* and do not exhibit any signs of disease (Polkinghorne, Hanger,

& Timms, 2013). Diagnosis of chlamydial disease in koalas has been by a combination of visual assessment of clinical signs of disease, ultrasonography of the koala urogenital tract, and, up until recently, a diagnostic point-of-care (POC) solid-phase enzyme immunoassay for detection of *Chlamydia* lipopolysaccharide antigen (Clearview®); however, this product is no longer commercially available. In addition, Hanger et al. (2013) showed that Clearview was only 43.2% sensitive and 92% specific compared with *C. pecorum* detection via qPCR, indicating that a Clearview result lacked specificity and sensitivity. With production of Clearview® ceasing, there is currently no alternative POC method for detection of *C. pecorum* infection in koalas available to wildlife hospitals, and consequently, there has been an increasing reliance on external diagnostic laboratories for confirmation of infection via molecular detection incorporating nucleic acid amplification tests such as conventional and real-time PCR (Hulse et al., 2018; Sachse et al., 2009).

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Polymerase chain reaction (PCR) is considered the “gold standard” diagnostic assay of choice for detection of *Chlamydia* based on its reliability and high sensitivity and specificity. However, a key disadvantage of PCR-based methods has been the restriction of its use to research and/or diagnostic laboratories to undertake sample processing and time-consuming thermocycling protocols, which can delay diagnosis and subsequent treatment by 24–48 hr. This means that infection status of koalas is not available to veterinarians and koala carers until well after initial decisions on animal management have been determined, leading to delays for application of animal therapeutic treatment regimes.

Molecular diagnostic tools that can be performed at POC have been developed incorporating loop-mediated isothermal amplification (LAMP), a newly developed, simple yet sophisticated, gene amplification method (Notomi, Mori, Tomita, & Kanda, 2015). The key advantage of LAMP is that it is a simple and rapid protocol, which means “on-the-spot” pathogen diagnosis is possible. It offers high sensitivity and specificity even when there is a decreased amount of target DNA available (Notomi et al., 2000). Using LAMP, the target sequence is amplified at a constant temperature of 60–65°C (no cycling required) using multiple sets of primers and a polymerase with high strand displacement activity in addition to a replication activity. Typically, four different primers are used to identify six distinct regions on the target gene, which adds highly to the specificity. An additional pair of “loop primers” further accelerates the reaction. Due to the specific nature of the action of these primers, the amount of DNA produced using LAMP is considerably higher than PCR-based amplification, meaning results are available in less than an hour. As confirmation of amplification of the correct target, an anneal step, consisting of 98–80°C at 0.01°C/s resulting in an anneal temperature of the amplicon produced, is performed after the isothermal step. Each target will have its own signature anneal temperature which is determined by the GC content of the generated amplicon. Positive amplification followed by an anneal temperature at the expected temperature is confirmation of a true positive in a sample.

The Genie® III (OptiGene Limited) is a compact, portable, battery-operated device for isothermal amplification and real-time fluorescence detection at the POC. LAMP assays performed on the Genie® III provide a rapid diagnostic tool with high specificity, due to the multiple primer sets; high sensitivity; all with minimal sample template preparation, which allows for easy incorporation as a diagnostic tool within the field or wildlife hospital, as previously reported by Jelocnik et al. (2017). In the present study, we describe the development and validation of a *C. pecorum*-specific LAMP assay on the Genie® III using conjunctival, urogenital, and rectal koala swabs, aimed at POC pathogen detection by wildlife hospital veterinary clinicians and koala ecologists in the field.

## 2 | MATERIALS AND METHODS

### 2.1 | Sample template preparation

Duplicate conjunctiva, urogenital sinus (females) or penile urethra (males), and rectal samples were collected from koalas

admitted to Currumbin Wildlife Hospital (Currumbin, Queensland) and Australia Zoo Wildlife Hospital (Beerwah, Queensland) using rayon-tipped aluminum-shafted swabs (Copan, Murrieta, California). Opportunistic sampling of koalas admitted to wildlife hospitals included animals exhibiting clinical signs of disease and symptoms unrelated to infectious disease (e.g., trauma, ill thrift, poor body condition).

Two crude DNA lysing methods to isolate DNA template from patient swabs were evaluated, as recommended by Optigene LAMP user guide for Mastermixes and assay optimization. The first method consisted of a two-step lysis procedure, whereby 0.2 ml of nuclease free water was added to a swab in a 2-ml microtube followed by vigorous mixing. A 50 µl aliquot was then heated to 95°C for 5 min to disrupt cell membranes and release cell contents into the buffer. A second method consisted of a crude one-step lysis method for the LAMP assay by the addition of 0.2 ml alkaline solution (potassium hydroxide, KOH) to a swab in a 2-ml microtube followed by vigorous mixing. The addition of KOH to the swab enables destruction of the cell wall of any bacteria or host cells present, releasing the cell contents, including bacterial DNA into the lysis buffer.

### 2.2 | Bacterial strains and DNA preparation

Assay specificity was determined by testing DNA extracts from 33 bacterial pathogens, including DNA extracted from additional *Chlamydia* sp. isolates to assess LAMP assay cross-reactivity and nontarget bacterial species identified as possible natural flora within the conjunctival and urogenital tract of koalas or close relatives of these bacterial species (See Table A1) (Blanshard, 1994; Blanshard & Bodley, 2008). Bacterial genomic DNA was extracted from isolates using the DNeasy Blood & Tissue Kit (QIAGEN Inc) according to manufacturer's instructions. In addition, DNA was isolated from quantified (inclusion-forming units; IFU/ml) reference *C. pecorum* strain culture and used as a positive control for the development and optimization of the LAMP assay. Assay sensitivity and limit of detection were determined by constructing a standard curve in the range of  $4.39 \times 10^1$ – $10^7$  (44–43,900,000 IFU/mL) using genomic DNA extracted from *C. pecorum* culture purified and quantified as described in Caldwell, Kromhout, and Schachter (1981).

### 2.3 | LAMP primer design

LAMP primers were designed from the *Chlamydia pecorum* P787, complete genome sequence (GenBank accession # CP004035) with the target primer sequences coding for the *C. pecorum* mreC gene, encoding for cell shape determining protein, with an amplicon length of 188 bp. Six oligonucleotide primers, consisting of the outer forward primer (F3), outer backward primer (B3), forward inner primer (FIP), backward inner primer (BIP), loop forward (LoopF), and loop backward (LoopB), were selected using licensed LAMP Designer 1.15 software (Premier Biosoft). Primer sequences were designed to specifically avoid cross homologies with other chlamydial species.

Intraspecies specificity of the target sequence was confirmed using the Basic Local Alignment Search Tool (BLAST) on the National Center for Biotechnology Information (NCBI) server (<http://www.ncbi.nlm.nih.gov/>) and Geneious Prime 2019.1.3. Primers were synthesized by Integrated DNA Technologies. Table 1 provides information on the name and sequence of each oligonucleotide primer, and Figure 1 presents the nucleotide position and target gene location of LAMP primers within the *C. pecorum* genome generated from Geneious Prime 2019.1.3.

## 2.4 | LAMP reaction and amplification condition

To make a comparative analysis and establish specificity of the LAMP assay by comparing amplification results with qPCR, published primers for two independent qPCR assays that targeted *C. pecorum* OmpA and OmpB gene sequences (Table 1) were used on DNA extracted (MagJET genomic DNA kit, Thermo Fisher Scientific) and purified (KingFisher Flex, Thermo Fisher Scientific) from the duplicate conjunctiva, urogenital sinus, and rectal clinical swab samples used for development of the LAMP assay (Hulse et al., 2018; Kollipara et al., 2013). *C. pecorum* OmpA and OmpB qPCR were performed on a RotorGene-Q real-time PCR cyclor (Qiagen) to validate LAMP assay results.

LAMP reaction was carried out using a total of 25 µl of the reaction mixture containing 5 µl of primer mix (Table 2), 15 µl of LAMP Isothermal Master Mix (Optigene), and 5 µl of sample. DNA lysis buffer was added as a template.

The LAMP assay was run at 65°C for 30 min with a melting curve analysis step (annealing curve 98°–80°C ramping at 0.01°C/s) on the portable Genie ® III (OptiGene) real-time fluorometer. A no template (water) control and DNA extracted from *C. pecorum* strain culture were used as negative and positive controls, respectively. Positive and negative results of samples were evaluated based on amplification plots, time to positivity, and melting curve and temperature of target amplicons analyzed automatically by the Genie III system.

**TABLE 1** Primer sets designed for *C. pecorum* LAMP and qPCR

Purpose	Primer name	Nucleotide sequence (5'–3')	Source	GenBank accession <sup>a</sup>
LAMP assay	F3	TATCGTGATCCTGCACATTG	This study	CP004035
	B3	CGCATTGCAATTACAGAAGG	This study	(6,925–7,112)
	FIP	TCCCGAAAGCACAGGAGAATTCGCTCGTGTTGGGTAGATG	This study	
	BIP	AAGGTGCTAGTTGGTCTTGTGGCTTCATGCCTTCATCCGTAA	This study	
	LoopF	TCTTTACTCCTTTGTCCTTCCC	This study	
	LoopB	AAGCAATCTCGGTACGGTT	This study	
qPCR	OmpB-F	CCAAGCATAATCGTAACAA	Hulse et al., 2018	U56924 (125–265)
	OmpB-R	CGAAGCAAGATTCTTGTC	Hulse et al., 2018	
	OmpB-Cy5	Cy5-ACTTGTGGCAATCTTCTCTTCACA	Hulse et al., 2018	
	OmpA-F	ATGAAAAAACTCTTAAAATCGG	Kollipara et al., 2013	NC_015408
	OmpA-R	TTAGAATCTGCATTGAGCAG	Kollipara et al., 2013	(56741–57916)

<sup>a</sup>Numbers in parentheses indicate nucleotide position.

## 3 | RESULTS

### 3.1 | Evaluation of sample template preparation

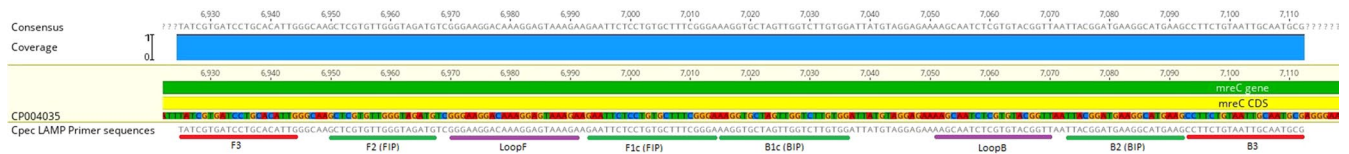
Comparison of sample template preparation revealed the heating method did not appear to sufficiently lyse the template, whereby no amplification of product occurred from samples confirmed positive via real-time PCR results. Establishing that the first method of sample template preparation was unsuccessful, the second method of sample template preparation was applied for the remainder of the study. The addition of KOH to swab samples sufficiently released template DNA into the buffer prior to LAMP testing, confirmed by comparison with real-time PCR results of the duplicate sample.

### 3.2 | *Chlamydia pecorum* limit of detection—Sensitivity

Table 3 presents the time to amplification of the *C. pecorum* standard curve and corresponding melt temperature. Linear regression of the standard curve determined the LAMP assay level of detection, established at 44 *C. pecorum* IFU/ml and amplifying in less than 20 min. Figure 2 graphically presents the amplification data of the standard curve generated from Genie Explorer V2.0.5.0 (OptiGene Limited).

### 3.3 | Comparative specificity of *C. pecorum* LAMP Assay

Specificity of the *C. pecorum* LAMP assay was demonstrated by amplification only occurring in DNA extracted from the *C. pecorum* isolate with no cross-hybridization with nontarget bacterial DNA (Table A1). A total of 43 DNA extracts from clinical samples were tested with two *C. pecorum* qPCR assays, each targeting different genes, and compared with LAMP assay results from the corresponding duplicate clinical swab, whereby sample template was prepared by the addition of KOH to the swab. Table 4 presents 100% agreement between the three methods of detection with Table A2 demonstrating



**FIGURE 1** *C. pecorum* LAMP assay primer sequences and nucleotide positions in the target gene regions. Outer F3 and B3 primers are shown in red; inner FIP and BIP in green; and LoopF and LoopB in purple

comparison with LAMP assay time to amplification with real-time PCR amplification cycling threshold for both qPCR assays.

## 4 | DISCUSSION

Rhodes et al. (2011) have suggested that chlamydial infection and disease, predominately attributed to *C. pecorum*, is a key agent of population decline of koalas in SEQLD. Previously, POC tests for identification of *C. pecorum* infections in koalas have relied on the Clearview® Chlamydia Antigen test, despite the poor sensitivity of the test as reported by Hanger et al. (2013). Thus, identification of *C. pecorum* infection in the koala is now predominately determined by nucleic acid amplification tests (e.g., Hulse et al., 2018). However, the use of these assays, which are highly sensitive and specific to the target pathogen, is generally restricted to research and diagnostic laboratories, a process that can be time-consuming and present delays to the veterinary clinician in diagnosis and animal management decisions.

Among the vast diversity of available nucleic acid amplification protocols, the technique of loop-mediated isothermal amplification can be considered a very promising application for POC systems for pathogen detection. The availability of a point-of-care LAMP assay, such as the one described and validated in this study, enables the clinician to provide a definitive diagnosis within an hour of clinical samples being collected and immediate application of appropriate therapeutic interventions.

In this study, we describe the design of a LAMP assay for the rapid detection of *C. pecorum* on a Genie® III and the evaluation of its performance against koala clinical samples and nontarget pathogens as a POC application. This study has shown that the *C. pecorum* LAMP assay is 100% sensitive and specific in detecting *C. pecorum* infection from koala clinical swab samples, when compared to the real-time PCR assays, with a level of detection at 44 *C. pecorum* IFU/ml. While it is uncommon for a diagnostic assay to

be 100% sensitive and specific compared with the gold standard method, it is highly likely that if there was more than one sample type being evaluated, there would be lower correlation between tests. However, this study evaluated the standard sample type collected from koalas for molecular detection of *Chlamydia* sp. within wildlife hospitals.

LAMP has successfully been used to diagnose various pathogens and viruses in humans and animals (Dhama et al., 2014), and due to its simplicity, robustness, and low cost, LAMP can be used as a simple screening assay in the field or at the point of care by clinicians. While Jelocnik et al. (2017) presented a similar study whereby LAMP assays were developed targeting *C. psittaci* and *C. pecorum* and were designed to run on a Genie® III as a diagnostic tool for both human and veterinary clinicians, this study has further developed the concept to establish a one-step method of swab sample preparation prior to LAMP testing, hence eliminating the heating step outlined in this study and the method presented by Jelocnik et al. (2017) further minimizing the additional use of labor and equipment. This study has demonstrated that cell lysis via KOH rapidly releases sufficient quantity of template DNA from crude samples, while minimizing the presence of amplification inhibitors and therefore the likelihood of false negatives occurring.

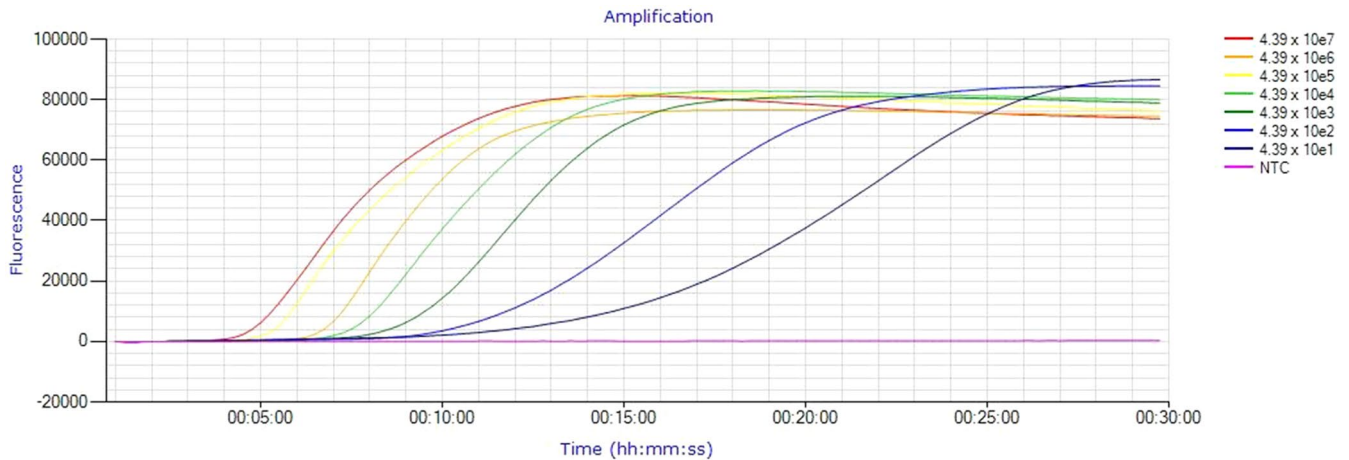
The methodology outlined in this study has already been successfully incorporated as a point-of-care diagnostic tool within Currumbin Wildlife Hospital, Queensland, RSPCA Wildlife, Wacol, Queensland, and Moggill Koala Rehabilitation Centre, Queensland, and used in the field for “on the spot” diagnosis of captured wild koalas. Senior veterinarian of Currumbin Wildlife Hospital, Dr Michael Pyne reported that following 6 months of using the *C. pecorum* LAMP assay on the Genie III platform as part of the hospital's in-clinic diagnostics, he is now able to rapidly and accurately assess hospitalized koalas for chlamydial

**TABLE 2** LAMP assay primer mix

Primer (25 pmol/μl)	Volume (μl)	Concentration (μmol/L)
F3	2.5	0.2
B3	2.5	0.2
LoopF	12.5	1.0
LoopB	12.5	1.0
FIP	25	2.0
BIP	25	2.0
Nuclease free H2O	170	

**TABLE 3** *C. pecorum* standard curve dilution series time to amplification and melt temperature

<i>C. pecorum</i> concentration (IFU/ml)	Time to amplification	Melt (°C)
$4.39 \times 10^7$	05:30	84.75
$4.39 \times 10^6$	07:30	84.59
$4.39 \times 10^5$	08:00	84.68
$4.39 \times 10^4$	08:15	84.55
$4.39 \times 10^3$	10:15	84.42
$4.39 \times 10^2$	13:00	84.71
$4.39 \times 10^1$	18:00	84.77
NTC	-	-



**FIGURE 2** Amplification of *C. pecorum* standard curve

**TABLE 4** Comparison of results from *C. pecorum* real-time PCR and LAMP assays using clinical swabs

Assay	Koala conjunctival swabs	Koala urogenital swabs	Koala rectal swabs
	Positive	Positive	Positive
<i>C. pecorum</i> OmpA (real-time PCR)	12/24	13/16	1/3
<i>C. pecorum</i> OmpB (real-time PCR)	12/24	13/16	1/3
<i>C. pecorum</i> LAMP	12/24	13/16	1/3

infection and make quick decisions on treatment options in subclinical cases, which allows for faster release times when assessing koalas post-treatment (M. Pyne, personal communication, 20 May 2019). In the field, rapid monitoring of the infectious status of koalas is important in an effort to better understand the prevalence and epidemiology of chlamydiosis, but this has currently not been possible due to current nucleic acid detection methods requiring 24–48 hr to perform in a diagnostic laboratory. The *C. pecorum* LAMP assay is a rapid test that can be performed in the field on freshly collected samples that require minimal processing and thereby be used to fast track conservation management decisions.

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#### CONFLICT OF INTERESTS

None declared.

#### AUTHOR CONTRIBUTIONS

Conceptualization: Ken Beagley and Lyndal Hulse; data curation, investigation, and formal analysis: Lyndal Hulse; funding acquisition: Stephen Johnston and Ken Beagley; methodology: Sean McDonald and Lyndal Hulse; writing—original draft preparation: Lyndal Hulse; writing—review and editing: Stephen Johnston, Ken Beagley, and Lyndal Hulse.

#### ETHICS STATEMENT

Samples were collected under the Department of Environment Scientific Purposes Permit WISP16751015 and University of Queensland Animal Ethics Permit AE02305.

#### DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article.

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## APPENDIX

**TABLE A1** Specificity of the *C. pecorum* LAMP assay against target and nontarget reference strains and isolates

Strain/isolate	<i>C. pecorum</i>	
	Time to amplification	Melt (°C)
<i>Mycoplasma gallisepticum</i> ts-11	-	-
<i>Mycoplasma synoviae</i> MS-H	-	-
<i>Ureplasma parvum</i> serovar 3 S34425 P4JN	-	-
<i>Ureplasma parvum</i> serovar 6 UPS6	-	-
<i>Ureplasma parvum</i> serovar 5 UPS5	-	-
<i>Ureplasma parvum</i> serovar 10 UPS10	-	-
<i>Chlamydia pecorum</i> (Mars Bar)	05:30	84.74
<i>Chlamydia trachomatis</i> serovar D	-	-
<i>Chlamydia trachomatis</i> serovar E	-	-
<i>Chlamydia muridarum</i>	-	-
<i>Chlamydia caviae</i>	-	-
<i>Chlamydia pneumoniae</i> A03	-	-
<i>Chlamydia pneumoniae</i> (koala)	-	-
<i>Bordetella bronchiseptica</i> BR977 (pig)	-	-
<i>Bordetella bronchiseptica</i> BR976 (koala)	-	-
<i>Bordetella bronchiseptica</i> BR978	-	-
<i>Bordetella hinzii</i> TC58T (=LMG 13501T) (chicken)	-	-
<i>Bordetella avium</i> 591-77T (=ATCC 35086T) (turkey)	-	-
<i>Bordetella bronchiseptica</i> TC42 (turkey)	-	-
<i>Bordetella bronchiseptica</i> TC6T (=ATCC 19395T) (dog)	-	-
<i>Staphylococcus aureus</i> BR256 (=ATCC 29213)	-	-
<i>Escherichia coli</i> BR316 (=ATCC 25922)	-	-
<i>Pseudomonas aeruginosa</i> BR317 (=ATCC 27853)	-	-
<i>Staphylococcus pseudintermedius</i> (dog)	-	-
<i>Escherichia coli</i> (dog)	-	-
<i>Streptococcus</i> gpG (dog)	-	-
<i>Pseudomonas aeruginosa</i> (dog)	-	-
<i>Pseudomonas aeruginosa</i> BR317 (=ATCC 27853)	-	-
<i>Staphylococcus pseudintermedius</i> (dog)	-	-
<i>Klebsiella pneumoniae</i> (human)	-	-
<i>Klebsiella pneumoniae</i> (horse)	-	-
<i>Klebsiella pneumoniae</i> (dog)	-	-
<i>Klebsiella pneumoniae</i> (koala)	-	-
<i>Klebsiella oxytoca</i> (human)	-	-
<i>Klebsiella oxytoca</i> (horse)	-	-

**TABLE A2** Specificity of *C. pecorum* LAMP assay against real-time PCR *C. pecorum* OmpA and OmpB target genes

Sample number	Koala swab sample	<i>C. pecorum</i> LAMP		<i>C. pecorum</i> OmpA (real-time PCR)	<i>C. pecorum</i> OmpB (real-time PCR)
		Time to amplification	Melt temperature (°C)	Cycling threshold	Cycling threshold
78,729	Left conjunctival	0:00	0.00	0	0
	Right conjunctival	10:00	84.97	23.22	29.06
	Urogenital	10:30	85.40	22.06	23.98
78,732	Left conjunctival	0:00	0.00	0	0
	Right conjunctival	0:00	0.00	0	0
	Urogenital	15:00	85.15	26.01	32.71
78,661	Urogenital	11:00	85.15	35.04	34.51
	Rectal	13:45	84.96	28.46	33.78
71,942	Left conjunctival	0:00	0.00	0	0
	Right conjunctival	0:00	0.00	0	0
	Urogenital	0:00	0.00	0	0
72,035	Urogenital	17:08	84.56	18.16	22.41
72,097	Left conjunctival	0:00	0.00	0	0
78,861	Pooled conjunctival	0:00	0.00	0	0
	Urogenital	10:45	85.07	25.23	33.09
78,822	Pooled conjunctival	13:45	84.76	24.31	25.51
	Urogenital	10:15	84.96	25.59	29.45
79,193	Pooled conjunctival	0:00	0.00	0	0
	Urogenital	0:00	0.00	0	0
	Rectal	0:00	0.00	0	0
71,499	Left conjunctival	9:30	84.51	24.36	28.57
	Right conjunctival	12:00	84.32	23.84	31.21
	Urogenital	16:30	84.61	31.43	30.31
71,494	Left conjunctival	0:00	0.00	0	0
	Urogenital	7:30	84.42	22.65	27.98
71,586	Left conjunctival	9:45	84.66	20.61	27.95
	Right conjunctival	14:00	84.77	22.59	27.31
	Urogenital	15:30	85.00	20.76	29.44
71,257	Left conjunctival	14:15	84.86	18.79	22.40
	Right conjunctival	7:15	84.97	17.98	20.66
	Urogenital	6:15	84.87	15.47	19.10
71,472	Left conjunctival	0:00	0.00	0	0
	Right conjunctival	0:00	0.00	0	0
	Urogenital	18:45	85.26	31.25	25.69
71,719	Left conjunctival	16:30	84.36	27.95	23.82
71,720	Left conjunctival	0:00	0.00	0	0
	Right conjunctival	17:03	84.70	30.55	32.40
	Urogenital	16:45	84.56	20.85	27.83
78,931	Left conjunctival	17:30	84.56	26.05	27.99
	Right conjunctival	12:45	84.17	24.29	27.00
	Urogenital	18:15	84.27	30.83	28.00
	Rectal	0:00	0.00	0	0
78,858	Urogenital	0:00	0.00	0	0