Development and evaluation of rapid novel isothermal amplification assays for important veterinary pathogens: *Chlamydia psittaci* and *Chlamydia pecorum*

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Background *Chlamydia psittaci* and *Chlamydia pecorum* are important veterinary pathogens, with the former also being responsible for zoonoses, and the latter adversely affecting koala populations in Australia and livestock globally. The rapid detection of these organisms is still challenging, particularly at the point-of-care (POC). In the present study, we developed and evaluated rapid, sensitive and robust *C. psittaci*-specific and *C. pecorum*-specific Loop Mediated Isothermal Amplification (LAMP) assays for detection of these pathogens.

Methods and materials The LAMP assays, performed in a Genie III real-time fluorometer, targeted a 263 bp region of the *C. psittaci*-specific Cps_0607 gene or a 209 bp region of a *C. pecorum*-specific conserved gene CpecG_0573, and were evaluated using a range of samples previously screened using species-specific quantitative PCRs (qPCRs). Species-specificity for *C. psittaci* and *C. pecorum* LAMP targets was tested against DNA samples from related chlamydial species and a range of other bacteria. In order to evaluate pathogen detection in clinical samples, *C. psittaci* LAMP was evaluated using a total of 26 DNA extracts from clinical samples from equine and avian hosts, while for *C. pecorum* LAMP, we tested a total of 63 DNA extracts from clinical samples from koala, sheep and cattle hosts. A subset of 36 *C. pecorum* samples was also tested in a thermal cycler (instead of a real-time fluorometer) using newly developed LAMP and results were determined as an end point detection. We also evaluated rapid swab processing (without DNA extraction) to assess the robustness of these assays.

Results Both LAMP assays were demonstrated to species-specific, highly reproducible and to be able to detect as little as 10 genome copy number/reaction, with a mean amplification time of 14 and 24 minutes for *C. psittaci* and *C. pecorum*, respectively. When testing clinical samples, the overall congruence between the newly developed LAMP assays and qPCR was 92.3% for *C. psittaci* (91.7% sensitivity and 92.9% specificity); and 84.1% for *C. pecorum* (90.6 % sensitivity and 77.4 % specificity). For a subset of 36 *C. pecorum* samples tested in a thermal cycler using newly developed LAMP, we observed 34/36 (94.4%) samples result being congruent between LAMP performed in fluorometer and in thermal cycler. Rapid swab processing method evaluated in this study also allows for chlamydial DNA detection using LAMP.

Discussion In this study, we describe the development of novel, rapid and robust *C. psittaci*-specific and *C. pecorum*-specific LAMP assays that are able to detect these bacteria in clinical samples in either the laboratory or POC settings. With further development and a focus on the preparation of these assays at

the POC, it is anticipated that both tests may fill an important niche in the repertoire of ancillary diagnostic tools available to clinicians.

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Running title: *C. psittaci* and *C. pecorum* LAMP assays

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Abstract

Background

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Methods and materials

The LAMP assays, performed in a Genie III real-time fluorometer, targeted a 263 bp region of the *C. psittaci*-specific Cps_0607 gene or a 209 bp region of a *C. pecorum*-specific conserved gene CpecG_0573, and were evaluated using a range of samples previously screened using species-specific quantitative PCRs (qPCRs). Species-specificity for *C. psittaci* and *C. pecorum* LAMP targets was tested against DNA samples from related chlamydial species and a range of other bacteria.

In order to evaluate pathogen detection in clinical samples, *C. psittaci* LAMP was evaluated using a total of 26 DNA extracts from clinical samples from equine and avian hosts, while for *C. pecorum* LAMP, we tested a total of 63 DNA extracts from clinical samples from koala, sheep and cattle hosts. A subset of 36 *C. pecorum* samples was also tested in a thermal cycler (instead of a real-time fluorometer) using newly developed LAMP and results were determined as an end

point detection. We also evaluated rapid swab processing (without DNA extraction) to assess the robustness of these assays.

Results

Both LAMP assays were demonstrated to be species-specific, highly reproducible and able to detect as little as 10 genome copy number/reaction, with a mean amplification time of 14 and 24 minutes for *C. psittaci* and *C. pecorum*, respectively. When testing clinical samples, the overall congruence between the newly developed LAMP assays and qPCR was 92.3% for *C. psittaci* (91.7% sensitivity and 92.9% specificity); and 84.1% for *C. pecorum* (90.6 % sensitivity and 77.4 % specificity). For a subset of 36 *C. pecorum* samples tested in a thermal cycler using newly developed LAMP, 34/36 (94.4%) samples were similar positive using the fluorometer. A rapid swab processing method evaluated in this study also allows for chlamydial DNA detection using LAMP.

Discussion

In this study, we describe the development of novel, rapid and robust *C. psittaci*-specific and *C. pecorum*-specific LAMP assays that are able to detect these bacteria in clinical samples in either the laboratory or POC settings. With further development and a focus on the preparation of these assays at the POC, it is anticipated that both tests may fill an important niche in the repertoire of ancillary diagnostic tools available to clinicians.

Introduction

The obligatory intracellular bacteria, *Chlamydia psittaci* and *Chlamydia pecorum*, are globally widespread veterinary pathogens that cause disease in an astonishing range of hosts. *C. psittaci*, the causative agent of psittacosis or wasting bird disease, is regarded as a major economically relevant poultry and pet bird pathogen (Knittler & Sachse 2015; Szymanska-Czerwinska & Niemczuk 2016). Globally, *C. psittaci* infections are also sporadically reported in other animal species such as pigs, cattle, sheep and horses, resulting in asymptomatic shedding, acute respiratory disease and, in the case of horses, reproductive loss (Reinhold, Sachse & Kaltenboeck 2011; Knittler & Sachse 2015; Jelocnik *et al.* 2017). Importantly, this pathogen continues to pose risks to public health through zoonotic transmission events that may lead to severe pneumonia (Gaede *et al.* 2008; Laroucau *et al.* 2015; Branley *et al.* 2016). This zoonotic risk is typically associated with direct contact with *C. psittaci* infected birds, although indirect contact through exposure to environmental contamination has been suggested (Branley *et al.* 2014; Branley *et al.* 2016).

C. pecorum is perhaps best known as the major pathogen of the iconic Australian native species, the koala. These infections are most commonly asymptomatic but can also result in serious inflammatory ocular and/or urogenital disease, affecting almost all Australia's mainland koala populations (Polkinghorne, Hanger & Timms 2013; Gonzalez-Astudillo *et al.* 2017). *C. pecorum* is also an important livestock pathogen causing a range of debilitating diseases such as sporadic bovine encephalomyelitis, polyarthritis, pneumonia and conjunctivitis, with faecal shedding as a constant feature of these infections (Lenzko *et al.* 2011; Reinhold *et al.* 2011; Walker *et al.* 2015). In livestock, chlamydial pathogens such as *C. pecorum* and *C. psittaci* may be found as co-infections, raising the possibility of a synergistic pathogenic effect (Lenzko *et al.* 2011;

Reinhold *et al.* 2011; Knittler & Sachse 2015). The reports of chlamydial infections in novel hosts and their recognised pathogenic potential (Jelocnik *et al.* 2015b; Burnard & Polkinghorne 2016; Taylor-Brown & Polkinghorne 2017), further highlight the need for faster detection and molecular discrimination of infecting strains.

Whilst significant progress has been made in understanding the molecular epidemiology of *C. psittaci* and *C. pecorum* infections (Jelocnik *et al.* 2015a; Branley *et al.* 2016;), the diagnosis and detection of these pathogens is still difficult, laborious and costly, challenging efforts to manage and treat infected hosts. A variety of traditional (cell culture, antigen detection, and serology) and molecular (conventional and real-time quantitative PCR (qPCR)) diagnostic options are used to detect chlamydial infections and diagnose chlamydiosis (Sachse *et al.* 2009). For both *C. psittaci* and *C. pecorum*, nucleic acid amplification tests (NAATs) are presently considered the diagnostic "gold standard" due to their specificity and sensitivity, however the use of these assays is mainly restricted to research and/or diagnostic laboratories. In the absence of standardised gene target(s) for these organisms, numerous single or nested species-specific qPCR assays have been proposed and/or are used for *C. psittaci* (Madico *et al.* 2000; Geens *et al.* 2005; Menard *et al.* 2006; Branley *et al.* 2008) and *C. pecorum* (Marsh *et al.* 2011; Higgins *et al.* 2012; Wan *et al.* 2011; Walker *et al.* 2016) diagnosis.

The development and use of low-cost molecular diagnostic tools performed at the point-of-care (POC) which fulfil the World Health Organization "ASSURED" criteria of affordable, sensitive, specific, user-friendly, rapid, equipment-free, and deliverable to those in need to be tested, are on

the exponential rise (Maffert *et al.* 2017). While POC testing is not necessarily required when considering most chlamydial infections of veterinary concern, the ability to provide a rapid detection of infections becomes of increasing significance when veterinarians and other animal workers may be at risk of being exposed to *C. psittaci* infections in field or farm settings. POC testing is also particularly relevant for *Chlamydia* screening in wild animals where laboratory testing is not accessible either due to logistics associated with field sampling or that services are not routinely available for testing of samples from wildlife. The latter problem is particularly acute for diagnosing infections in koalas, with the recent decision to stop the production of a commercially viable solid-phase ELISA leaving wildlife hospitals unable to diagnose and successfully treat asymptomatic *C. pecorum* infections (Hanger *et al.* 2013).

While there are many options for molecular POC diagnostics, Loop Mediated Isothermal Amplification (LAMP) assays developed for use in pathogen diagnostics are popular as they offer significant advantages over PCR and/or serology testing (Maffert *et al.* 2017). Rapid, simple, highly specific, easy to interpret, and carried out at a constant temperature, LAMP assays can provide a diagnosis in 30 min, in either laboratory or field setting (Mansour *et al.* 2015; Notomi *et al.* 2015). Rapid isothermal LAMP assays that could be performed at the POC targeting human *C. pneumoniae* (Kawai *et al.* 2009) and *C. trachomatis* (Jevtusevskaja *et al.* 2016; Choopara *et al.* 2017) infections have been proposed for use in chlamydial diagnostics. Development of a *C. pecorum* LAMP, in particular, would meet immediate demand for koala *C. pecorum* infections diagnostics, providing an alternative solution for the current laboratory diagnostics. A recent outbreak of psittacosis in veterinary staff and students in contact with a *C. psittaci*-infected and sick neonatal foal (Chan *et al.* 2017; Jelocnik *et al.* 2017), further

126 demonstrates the need for POC assays such as LAMP to rapidly diagnose *C. psittaci*. In the
127 present study, we describe the development and evaluation of rapid and robust *C. psittaci*-
128 specific and *C. pecorum*-specific LAMP assays for detection of these organisms in either
129 laboratory or POC settings.

130

131 **Materials and methods**

132 *Bacterial cultures and clinical samples used in this study*

133 *C. psittaci* LAMP assay was evaluated using: 1) 12 DNA samples extracted from previously
134 characterised *C. psittaci* isolates (10 human, two parrot and one equine) (Table S1); 2) DNA
135 extracted from 21 placental, foetal, nasal, lung and rectal swabs, and 1 each placental and foetal
136 tissue sample taken from 20 equine hosts; and 3) three pigeon liver DNA extracts (Table S2).
137 All samples were collected and submitted as part of routine diagnostic testing by field or district
138 veterinarians to the State Veterinary Diagnostic Laboratory (SVDL), Elizabeth Macarthur
139 Agricultural Institute (EMAI), Menangle, NSW, Australia, and as such does not require special
140 animal ethics approval. DNA extracts from these samples were kindly provided by Dr. Cheryl
141 Jenkins, and Dr. James Branley. The use of these swabs was considered by the University of The
142 Sunshine Coast (USC) Animal Ethics Committee and the need for further ethics consideration
143 was waived under exemption AN/E/17/19.

144 *C. pecorum* LAMP was evaluated using a: 1) 18 DNA samples extracted from previously
145 characterised koala (n=7), sheep (n=4), cattle (n=4) and pig *C. pecorum* (n=3) cultures (Table
146 S1); 2) 16 sheep and 13 cattle ocular, rectal, and tissue swab DNA samples; and 3) 34 ocular and
147 urogenital (UGT) koala swab DNA samples (Table S3), all available in our collection. The use

148 of these swabs, also collected by qualified veterinarians as a part of routine diagnostic testing,
149 was considered and approved for exemption by the University of The Sunshine Coast (USC)
150 Animal Ethics Committee (AN/E/14/01 and AN/E/14/31).

151

152 We also evaluated the specificity of the assays against DNA samples extracted from previously
153 characterised i) chlamydial isolates (koala C. *pneumoniae* LPColN, *C. abortus* S26/3, *C. suis*
154 S45, *C. trachomatis* serovar D, *C. murridarum* Nigg, *C. caviae* GPIC) and uncultured
155 *Chlamydiales* (*Fritschea* spp.); ii) Gram negative *Escherichia coli* and *Prevotella bivia*; Gram
156 positive *Fusobacterium nucleatum*, *Staphylococcus epidermidis*, *S. aureus*, *Streptococcus* spp.,
157 and *Enterococcus faecalis*; and iii) commercially available human gDNA (Promega, Alexandria,
158 NSW 2015), all available in our laboratory (Table S1).

159

160 In order to evaluate rapid swab processing, 18 ocular, cloacal and UGT (14 dry and four RNA-
161 Later) clinical swabs taken from 14 koalas with presumptive chlamydiosis were used for testing
162 without DNA extraction. Briefly, RNA-Later and dry swabs with added 500 µL TE buffer were
163 vortexed vigorously for 5 min. 300 µL aliquots were then heated to 98°C for 15 min to lyse
164 DNA, following LAMP testing. The use of these swabs, collected as a part of routine diagnostic
165 testing, is also under Animal Ethics approval exemption (AN/E/14/01). An aliquot of 50 µL of
166 the swab suspension was used for LAMP and qPCR assays, while from the remaining volume of
167 the swab suspension was used for DNA extraction, in order to compare swab suspension and its
168 paired extracted DNA as a template in the assays.

169

LAMP assays design

For the *C. psittaci*-specific LAMP gene target, we targeted a previously described conserved single-copy *C. psittaci*-specific CDS, encoding for hypothetical protein and denoted *Cpsi*t_0607 in the representative *C. psittaci* 6BC strain (Genbank accession number NC_015470.1) (Voigt, Schöfl & Saluz 2012). This gene was also previously proposed as a target for molecular diagnosis of *C. psittaci* infections (Opota *et al.* 2015).

The *C. pecorum*-specific candidate LAMP gene target, encoding for a single-copy conserved hypothetical protein and denoted CpecG_0573 in the *C. pecorum* MC/Marsbar koala type strain (Genbank accession number NZ_CM002310.1), was selected based on a comparative genomics analysis of published koala and livestock *C. pecorum* genomes (Jelocnik *et al*. 2015a). For the purposes of this study, we will refer to it as *Cpec*_HP. Both candidate gene sequences were aligned to the corresponding allele from other publicly available *C. psittaci* or *C. pecorum* strains using Clustal X (as implemented in Geneious 9 (Kearse *et al.* 2012)), and analysed in blastn against the nucleotide collection nr/nt database to assess intra-species sequence identity, and inter-species specificity.

For *C. ps*_0607 alignment, besides 6BC, we used the gene alleles from strains 84/55 (CP003790.1), 02DC15 (CP002806.1), 01DC11 (CP002805.1), WC (CP003796.1), 01DC12 (HF545614.1), NJ1 (CP003798.1), CR009 (LZRX01000000), Ho Re upper (LZRE01000000) and PoAn (LZRG01000000). For *C. pec*_HP alignment, besides MC/Marsbar, we used the gene alleles from E58 (CP002608.1), P787 (CP004035.1), W73 (CP004034.1), IPA (NZ_CM002311.1), NSW/Bov/SBE (NZ_JWHE00000000.1), L71 (LFRL01000000), L17

192 (LFRK01000001), L1 (LFRH00000000), DBDeUG (NZ_CM002308.1), SA/K2/UGT
193 (SRR1693792), Nar/S22/Rec (SRR1693794) and Mer/Ovi1/Jnt (SRR1693791).

194

195 Species-specific LAMP primers were designed using the target sequences with the open-source
196 Primer Explorer v5 software (Eiken Chemical Co., Japan) and licensed LAMP Designer 1.15
197 software (Premier Biosoft, USA). For both *C. pecorum* and *C. psittaci*, Primer Explorer v5
198 yielded five sets of four LAMP primers including two outer (forward F3 and backward B3)
199 primers and two inner (forward inner FIP and backward inner BIP) primers targeting different
200 regions of the target gene, while LAMP Designer yielded single best set of six LAMP primers
201 including two outer primers (forward F3 and backward B3), two inner primers (forward inner
202 FIP and backward inner BIP) and two loop primers (forward loop LF and backwards loop LB).
203 All primers (as single or paired) were tested *in silico*, including analysing primer sequences in
204 blast for species specificity and OligoAnalyser 3.1 (available from
205 http://sg.idtdna.com/calc/analyzer) for primer dimerization, hairpins and melting temperatures.

206 After *in silico* and in LAMP reaction testing, a set of four primers designed by PrimerExplorer
207 v5 and targeting a 209bp region of the *C. pec*_HP gene (spanning from position 22 to 230) was
208 selected for *C. pecorum* LAMP assays performed in this study. Additional loop primers (LF/LB)
209 were also designed to accelerate amplification time and increase sensitivity. For *C. psittaci*, a set
210 of six primers designed with LAMP Designer and targeting a 263bp region of the *C. ps*_0607
211 gene (spanning from position 286 to 548) was selected for LAMP assays performed in this study.
212 The specificity of primer sequences was assessed *in silico* using discontiquousBLAST analyses.
213 Amplicons generated by conventional PCR using outer F3 and B3 primers for both *C. psittaci*
214 and *C. pecorum* were gel excised, purified using Roche High Pure purification kit, and sent to

Australian Genome Research Facility (AGRF) for Sanger sequencing for sequence identity confirmation.

LAMP assay optimisation

Both *C. psittaci* and *C. pecorum* LAMP assays were carried out in a 25 µL reaction volume. The reaction mixture consisted of 15 µL Isothermal Master Mix ISO001 (Optigene, UK), 5 µL six primers mix (at 0.2 µM F3 and B3, 0.8 µM FIP and BIP, and 0.4 µM LF and LB) and 5 µL template, following LAMP assay run at 65°C in the Genie III real-time fluorometer (Optigene, UK), as per manufacturer instructions. Following determination of the most optimal conditions (fastest amplification time, fluorescence and annealing temperature), *C. psittaci* LAMP assays were run at 65°C for 30 min followed by annealing step of 98 – 80°C at a rate of 0.05°C /s, while *C. pecorum* LAMP assays were run using the same temperature and annealing conditions, however for 45 min. A negative control (LAMP mix only) was included in each run. Both *C. psittaci* and *C. pecorum* LAMP assays were performed on a thermal cycle heating block at 65°C for 30 min, following detection of amplicons by electrophoresis on a 1.5% ethidium bromide agarose gel and visualisation under UV. In addition, several *C. pecorum* LAMP assays were conducted using the four primer set, two outer (F3 and B3) and two inner (FIP and BIP) primers, on a heating block at 65°C for 45 min.

After the assay optimisation, LAMP testing was evaluated using previously tested clinical samples, previously characterised isolates and untested new samples. *C. pecorum*-presumptive samples were simultaneously tested using our in-house *C. pecorum*–specific qPCR assay (Marsh

237 *et al.* 2011), while *C. psittaci*-presumptive samples were tested using a pan-*Chlamydiales* qPCR
238 assay with primers 16SIGF and 16SIGR targeting the 298bp 16S rRNA fragment (Everett, Bush
239 & Andersen 1999). Amplicon sequencing was used for the latter assay to confirm species
240 identity. The qPCR assays were carried out in a 20 µL total volume, consisting of 10 µL
241 SYBR™ Green PCR Master Mix (Life Technologies Australia Pty Ltd., Scoresby, Vic 3179), 1
242 µL of each 10 µM forward and reverse primer, 3 µL miliqH2O, and 5 µL DNA template. The
243 qPCR assays were run for 35 cycles (Ct), and in each qPCR assay a positive (cultured *C.*
244 *pecorum* and/or *C. psittaci* DNA) and negative (miliqH2O) controls were included. Based on the
245 qPCR standard curve and the number of running cycles, samples amplifying at > 30 Ct (and/or
246 equivalent detected genome copy number) were considered negative. The 23 *C. psittaci*-
247 presumptive equine samples were also tested with a *C. psittaci*-specific qPCR assay targeting the
248 16S rRNA gene/16S-23S rRNA spacer gene (Madico *et al.* 2000) at the State Veterinary
249 Diagnostic Laboratory (SVDL), Elizabeth Macarthur Agricultural Institute (EMAI), Menangle,
250 NSW, Australia. Samples amplifying at > 39 Ct were considered negative. LAMP testing was
251 performed in a blind fashion, by two different operators, unaware of qPCR results.

252

253 *Statistical analyses*

254 For each assay, we compared the performance of two tests evaluated in the same population by
255 calculating Kappa and overall agreement, as well as estimated sensitivity and specificity (with
256 specified Clopper-Pearson (exact) confidence limits) of LAMP compared to the known
257 reference (gold standard) qPCR test using EpiTools online (Sergeant 2017). It is suggested the
258 Kappa value be interpreted as follows: values ≤ 0 as indicating no agreement and 0.01–0.20 as

none to slight, 0.21–0.40 as fair, 0.41– 0.60 as moderate, 0.61–0.80 as substantial, and 0.81–1.00 as almost perfect agreement.

Results and discussion

With the emergence of new spill-over threats posed by *C. psittaci* (Laroucau *et al.* 2015; Jelocnik *et al.* 2017), there is an increasing need for rapid diagnostic tools for this pathogen, particularly for those that may have practical application in the field or clinical setting. There are specific needs for *C. pecorum* POC tests as well in both the veterinary care and treatment of infected domesticated and native animals, particularly in settings where veterinary diagnostic testing is logistically challenging. In the present study, to the best of our knowledge, we describe the first development of novel, rapid and robust *C. psittaci*-specific and *C. pecorum*-specific LAMP assays that are able to detect these bacteria in clinical samples in either the laboratory or POC settings.

C. psittaci and *C. pecorum* LAMP development

A *C. psittaci*-specific gene (*C.ps*_0607) was previously characterised as a conserved gene sequence present only in *C. psittaci* genomes, and absent from all other related chlamydial species (Voigt *et al.* 2012). BLAST analyses and alignment of the *C.ps*_0607 gene sequences, including those from recently described human, bird and equine Australian isolates, confirmed species specificity and sequence conservation. Between 0 and 13 single nucleotide polymorphisms (SNPs) were observed amongst strains (100-95.1% sequence identity) based on a 263 bp alignment of *C.ps*_0607 gene sequences, including that from the most distant *C. psittaci*

NJ1 taxon (Figure S1A). Similarly, the *C. pecorum* HP gene (denoted CpecG_0573 locus in Marsbar strain) was determined as a highly conserved species-specific sequence following BLAST analysis against publicly available sequences. Using an alignment of HP gene sequences from 14 publicly available *C. pecorum* genomes, there were only 2 SNPs in the 209bp region to be targeted by LAMP (Figure S1B).

Although multiple LAMP primer sets were predicted, LAMP primer sets denoted in Figure 1 were chosen for further assay development. For *C. psittaci* assays, a set designed using LAMP Explorer was utilised while, for *C. pecorum*, we used a set designed with PrimerExplorer (Table 1). After initial testing, some of the predicted primer sets were discarded due to i) potential cross-amplification associated with a lack of specificity of the target primer; (ii) not achieving an amplification signal in the fluorometer; and iii) amplifying non-specific targets, including positive amplification in negative controls (data not shown). While we achieved initial amplification of a *C. psittaci* single copy dilution in a 30 min assay using the designed LAMP primer set, initial reaction times for a *C. pecorum* single copy amplification averaged 50 min. In order to accelerate amplification times for *C. pecorum*, we additionally designed a pair of Loop primers for the *C. pecorum* set which decreased the amplification of a single copy to 30 min.

Species-specificity for *C. psittaci* and *C. pecorum* LAMP targets was tested in the developed LAMP assays using DNA extracts from 12 *C. psittaci* and 18 *C. pecorum* cultured isolates, DNA extracts from other chlamydial species and a range of DNA extracts from other bacteria. Positive amplification as assessed by the presence of an observable amplification curve characterised by a

303 specific melt was observed only for the target species in their respective assays (Table S1). No
304 amplification curves were observed for any of the non-targeted chlamydial species or other
305 bacteria included in our specificity assays (Table S1). The *C. pecorum* and *C. psittaci* LAMP
306 assays did not amplify either the related chlamydial species or other bacteria included in our
307 specificity assays. In this study, in contrast, a previously described "*C. pecorum*-specific" qPCR
308 assay (Marsh *et al.* 2011; Wan *et al.* 2011) showed positive amplification and melt for *C.*
309 *psittaci* and *C. pneumoniae* DNA samples.

310 The choice to use the *C. ps*_0607 gene as a LAMP target was straight forward since it had been
311 suggested for such a purpose in previous studies (Voigt *et al.* 2012; Opota *et al.* 2015), For *C.*
312 *pecorum*, however, we utilised our ongoing comparative genomics to select *C. pecorum*-specific
313 and conserved *C.pec*_HP gene described in this study for the first time. *In silico* analyses and
314 assay development confirmed species-specificity of this gene and its suitability for use in
315 diagnostic assays. Previously published *C. pecorum* diagnostic assays targeted highly
316 polymorphic genes such as *omp*A (Higgins *et al.* 2012; Yang *et al.* 2014), which may require the
317 use of probes due to sequence variation, prolonging the detection time and increasing diagnostic
318 costs. Our routinely used in house *C. pecorum*-specific assay which targets a 204 bp 16S rRNA
319 fragment (Marsh *et al.* 2011; Wan *et al.* 2011) was simpler to use, however we have shown that
320 this assay may cross-react with other related chlamydial species due to a lack of sufficient
321 sequence variation in the region of the 16S rRNA gene targeted (Bachmann, Polkinghorne &
322 Timms 2014). For koala diagnostics where *C. pecorum* is the most abundant and prevalent
323 chlamydial organism (Polkinghorne *et al.* 2013), this cross-reactivity may not be of a big
324 concern. For the veterinary diagnosis of infections in livestock where co-infections with several
325 chlamydial species are common (Lenzko *et al.* 2011; Reinhold *et al.* 2011), this assay may be

less suitable. Using the *C. pecorum*-specific HP gene as a target in different diagnostic assays would hence seem promising.

Performance of the *C. psittaci* and *C. pecorum* LAMP assays

The sensitivity of the LAMP assays was evaluated using 5µL cultured *C. psittaci* and *C. pecorum* gDNA in 10-fold serial dilutions as a template in assays performed in triplicate in separate runs. The limits of detection of the LAMP assays were conservatively 10 copies for *C. psittaci*, with 3/3 (100%) positive amplification for 10 copy dilutions for *C. psittaci*, and 1 copy for *C. pecorum*, with 3/3 (100%) positive amplifications for a single copy dilution of *C. pecorum* DNA (Table 2 and 3). In the final and optimised LAMP assays, the mean amplification time detecting the lower limit (a single copy) for *C. psittaci* was 14.23 min with an average 84.45°C melt (Table 2) while, for *C. pecorum,* it was 24 min with an average 83.42°C melt (Table 3). Comparing the two newly developed assays, *C. psittaci* LAMP had the faster run time than that of *C. pecorum* LAMP. This difference in assays kinetics could be attributed to the improved *C. psittaci* LAMP primers design, as they were predicted by the LAMP Designer software (Nagamine, Hase & Notomi 2002). As we additionally designed Loop primers for *C. pecorum*, we can anticipate an improvement in the *C. pecorum* assay kinetics by re-designing the loop primers (e.g. extending the sequence to 20 – 22 bp), as well as testing LAMP mixes in different ratios and with improved polymerases.

In order to test the reproducibility of our LAMP assays, we tested a subset of *C. pecorum* and *C. psittaci* PCR positive samples (Table 4). All samples were run in a "blind fashion", in triplicate

348 and in separate runs by two different operators. For both assays, the amplification times and
349 melts of each sample between the runs were very similar, with 0 to 1.5 min (SDs ranging from 0
350 – 0.98) difference in amplification times for each sample, and 0.03 to 0.83°C (SDs ranging from
351 0.02 – 0.26) difference in melt for each sample. Congruence between the runs performed by
352 different operators indicates that both LAMP assays described in this study are highly
353 reproducible, and can detect the target organism in less than 30 min even when in low infectious
354 loads of < 10 copies.

355

356 Pathogen detection in clinical samples using newly developed LAMP

357 For *C. psittaci*, a total of 27 DNA extracts from clinical samples were tested with both *C. psittaci*
358 LAMP and qPCR assays (Table S2). For these analyses, samples with >20 min amplification
359 time were considered negative for LAMP, while for qPCR, samples with <20 genome
360 copy/reaction and/or > 30 Ct (quantification cycle) were considered negative, based on the qPCR
361 standard curve and the number of running cycles used for this testing. As observed in Table S2
362 and based on above cut-off values, 24/26 (92.3%) samples were congruent between the two tests,
363 with 11 samples positive and 13 samples negative by both (Table 5). For 2/26 (7.7%) where
364 there was disagreement, one sample was LAMP positive but qPCR negative, and another was
365 qPCR positive but *C. psittaci* LAMP negative. Based on these results, the Kappa value was
366 calculated at 0.85 (95% CI 0.64 – 1.05) indicating an almost perfect agreement between the tests.
367 The overall sensitivity of the *C. psittaci* LAMP was 91.7% (Clopper-Pearson 95% CI 0.62 –
368 0.99) and with 92.9% (Clopper-Pearson 95% CI 0.66 – 0.99) specificity, compared to the qPCR
369 used in this study. In addition, a subset of 23 samples was also tested independently by a third

370 party. Using a cut off of > Ct 39 as negative, 19/23 (82.60 %) of these test results were in
371 congruence with our *C. psittaci* LAMP results (Table S2).

372

373 For *C. pecorum*, we tested a total of 63 DNA extracts from clinical samples from several animal
374 hosts by both LAMP and qPCR (Table S3). For these analyses, samples with > 30 min
375 amplification time were considered negative for LAMP, while for qPCR, samples with < 35
376 genome copy /reaction and/or > 30 Ct were considered negative based on the standard curve and
377 number of run cycles used for this testing. For the 63 clinical samples, the overall congruence
378 was 84.1% with a Kappa value of 0.68 (95% CI 0.50 – 0.86), indicating substantial agreement
379 between the tests. Congruent results between tests were obtained for 53 samples, while there
380 were 10 discrepant samples using the above cut off for *C. pecorum* (Table 6). The overall
381 sensitivity of *C. pecorum* LAMP was 90.6 % (Clopper-Pearson 95% CI 0.75 – 0.98), while
382 specificity was 77.4 % (Clopper-Pearson 95% CI 0.59 – 0.90) in comparison to the qPCR assay.
383 A subset of 36 *C. pecorum* samples was also tested in a thermal cycler using the newly
384 developed LAMP and results were determined as an end point detection. For this experiment,
385 34/36 (94.4%) samples were congruent between LAMP performed in fluorometer and in a
386 thermal cycler (Table S3), demonstrating the robustness of the *C. pecorum* LAMP (Figure S2).

387

388 Considering that the qPCR assay used in this study to quantify and detect *C. psittaci* is
389 chlamydial genus rather species specific (Everett *et al.* 1999), high congruence observed for *C.*
390 *psittaci* assays could be attributed to testing a limited set of samples taken from hosts with
391 presumptive *C. psittaci* chlamydiosis. Lower congruence between the *C. pecorum*-specific

assays could be due to technical and experimental aspects and characteristics (such as the assay efficiency, analytical sensitivity, template preparation) (Bustin *et al.* 2010) of the *C. pecorum* 16S qPCR assay used in this study. As a sidenote, we also evaluated the use of *C. psittaci* and *C. pecorum* LAMP targets (263bp of the *C. ps*_0607 and 209bp *C. pec*_HP genes, respectively) using outer F3 and B3 primers in a fluorescence-based (SybrGreen) qPCR assays, if needed to estimate infectious loads of the pathogen. In this preliminary analyses, both targets seem suitable for use in qPCR assays as well, as we were able to detect low infectious load up to 10 copies/reaction in a sample.

Rapid swab processing

Rapid swab processing and using the swab suspension directly in LAMP assays were previously successfully evaluated for testing for respiratory syncytial virus from nasopharyngeal swabs (Mahony *et al.* 2013) and rapid detection of *Streptococcus agalactiae* in vaginal swabs (McKenna *et al.* 2017). A recent study also demonstrated that *C. trachomatis* can be detected directly from urine samples using the LAMP method (Jevtusevskaja *et al.* 2016). In this study, we also evaluated rapid swab processing without DNA extraction in order to begin to assess the POC potential of these assays. A total of 18 swabs taken from conjunctival and urogenital sites from koalas with presumptive chlamydiosis, of which four were stored in RNA Later and 14 were dry, were used for this experiment (Table 7). Vigorously vortexed and heated swab suspension samples were directly used as a template in both *C. pecorum* LAMP reaction performed in fluorometer and qPCR assay. We also performed DNA extraction from the swabs to be used as a comparison to rapid swab processing. We did not detect *C. pecorum* DNA in any of the RNA Later suspensions either by LAMP nor qPCR assay (Table 7), in contrast to

detecting *C. pecorum* in 50% (2/4) of the DNA extracts from the swabs by both methods. Using the rapidly processed swab suspension as a template, *C. pecorum* was detected in 6/14 by LAMP, and only 2/14 by qPCR (Table 7). The swab suspension LAMP results were 92.8% (13/14) congruent to the LAMP results and 85.7% congruent (12/14) to the qPCR results using the swab's paired DNA sample. In order to evaluate the potential presence of inhibitors in our samples, we "spiked" negative swab suspensions and its paired DNA samples with known amounts of *C. pecorum* (1x10^4 copies/reaction). As observed in Table 7, we detected *C. pecorum* by both LAMP and qPCR in "spiked" negative samples derived from dry swabs. No *C. pecorum* was detected in "spiked" RNA Later swab suspension, indicating the potential presence of inhibitors in these reactions. Our results suggest that the LAMP assays are capable of amplifying specific amplification products from crude DNA extracts.

Further work is additionally required to enhance the POC capabilities of these new chlamydial LAMP assays to meet the clinical need including (i) the evaluation of rapid swab processing methods using commercially available DNA release portable devices and/or sample preparation using microfluidic support; (ii) alternative amplification detection methods such as visible colorimetric or turbidimetric change and/or solid-phase 'dipstick' tests (Maffert *et al.* 2017). With further development and the aforementioned focus on the preparation of these assays at the POC (Parida *et al.* 2008; Tomita *et al.* 2008), it is anticipated that both LAMP tests described in this study may fill an important niche in the repertoire of ancillary diagnostic tools available to clinicians.

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Figure 1

LAMP primer sequences and positions in the target gene regions.

A) *C. psittaci* LAMP primer set; and B) *C. pecorum* LAMP primer set. Outer F3 and B3 primers are indicated in green, inner FIP and BIP in blue, and loop LF and BL in pink colour.

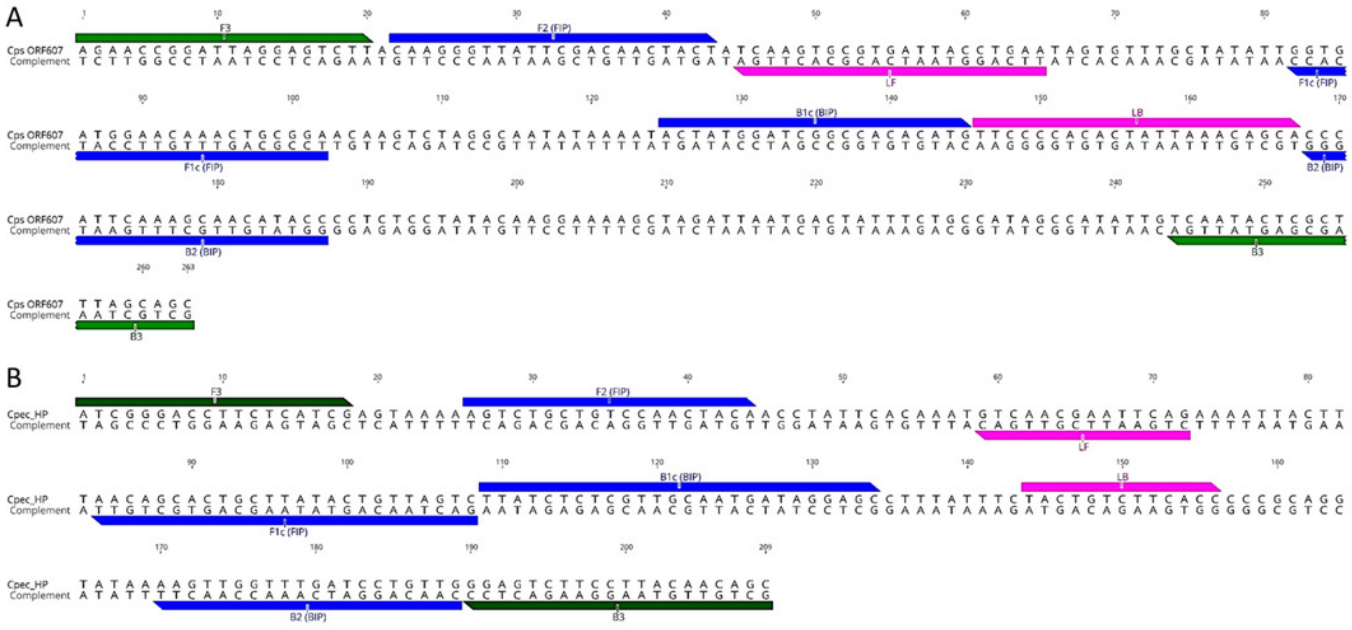

Table 1(on next page)

LAMP primers set used in this study.

1 **Table 1. LAMP primers set used in this study.**

C. psittaci LAMP primers			
Name	**Sequence 5' – 3'**	**Position**	**Length**
F3	AGAACCGGATTAGGAGTCTT	286	20
B3	GCTGCTAAAGCGAGTATTGA	548	20
FIP(F1c+F2)	TCCGCAGTTTGTTCCATCACCCAAGGGTTATTCGACAACTACT		43
BIP(B1c+B2)	ACTATGGATCGGCCACACATGGGTATGTTGCTTTGAATGGG		41
LoopF	TTCAGGTAATCACGCACTTGA	350	21
LoopB	TTCCCCACACTATTAAACAGCA	431	22
F2	CAAGGGTTATTCGACAACTACT	307	22
F1c	TCCGCAGTTTGTTCCATCACC	387	21
B2	GGTATGTTGCTTTGAATGGG	472	20
B1c	ACTATGGATCGGCCACACATG	410	21
***C. pecorum* LAMP primers**			
Name	**Sequence 5' – 3'**	**Position**	**Length**
F3	ATCGGGACCTTCTCATCG	22	18
B3	GCTGTTGTAAGGAAGACTCC	230	20
FIP(F1c+F2)	GACTAACAGTATAAGCAGTGCTGTTAGTCTGCTGTCCAACTACA		44
BIP(B1c+B2)	TTATCTCTCGTTGCAATGATAGGAGCCAACAGGATCAAACCAACTT		46
LoopF	CTGAATTCGTTGAC	93	14
LoopB	TACTGTCTTCACC	165	12
F2	AGTCTGCTGTCCAACTACA	47	19
F1c	GACTAACAGTATAAGCAGTGCTGTT	129	25
B2	CAACAGGATCAAACCAACTT	210	20
B1c	TTATCTCTCGTTGCAATGATAGGAGC	130	26

2

Table 2(on next page)

C. psittaci LAMP assay[a] sensitivity.

Table 2. *C. psittaci* LAMP assay[a] sensitivity.

Dilution*	Time to amplify (min)	Melt (°C)	Time (Mean + SD)	Melt (Mean + SD)
10^6	5.15	84.43		
10^6	5.00	84.46	5.10, 0.09	84.49, 0.08
10^6	5.15	84.58		
10^5	6.30	84.34		
10^5	6.45	84.33	6.30, 0.15	84.37, 0.06
10^5	6.15	84.43		
10^4	7.15	84.59		
10^4	7.30	84.58	7.25, 0.09	84.56, 0.04
10^4	7.30	84.51		
10^3	8.45	84.46		
10^3	8.15	84.43	8.25, 0.173	84.44, 0.01
10^3	8.15	84.44		
100	9.15	84.48		
100	9.30	84.39	9.30, 0.15	84.46, 0.06
100	9.45	84.51		
10	12.00	84.41		
10	11.00	84.35	11.33, 0.58	84.38, 0.03
10	11.00	84.39		
1	16.00	84.44		
1	0.00	0	14.23, 2.51	84.34, 0.14
1	12.45	84.24		
0.1	25.25	84.20		
0.1	-[b]	-	-	84.20
0.1	-	84.20[c]		

[a] The assay was performed in Genie III Real-time fluorometer, with the amplification times and annealing temperatures recorded at the end of each run. The samples were tested in three different runs; *Template was serially diluted *C. psittaci* CR009 gDNA which genome copy number was determined by qPCR. [b]: No amplification detected; [c]: No amplification, but melt and annealing curve recorded.

Table 3(on next page)

C. pecorum LAMP assay[#] sensitivity.

[#] The assay was performed in Genie III Real-time fluorometer, with the amplification times and annealing temperatures recorded at the end of each run. The samples were tested in different runs; *Template was serially diluted *C. pecorum* gDNA which genome copy number was determined by qPCR; from [a]: koala Marsbar isolate; [b]: sheep IPA isolate; and [c]: cattle E58 isolate; [d]: No amplification, but melt and annealing curve recorded.

Table 3. *C. pecorum* LAMP assay[#] sensitivity.

Dilution*	**Time to amplify** (min)	**Melt** (°C)	**Time** (Mean + SD)	**Melt** (Mean + SD)
10^7k[a]	10.00	83.23	10.23, 0.32	83.30, 0.1
10^7k	10.45	83.37		
10^6k	13.15	83.57		
10^6s[b]	13.15	83.33	12.92, 0.40	83.51, 0.16
10^6c[c]	12.45	83.62		
10^5k	14.00	83.52		
10^5s	14.00	83.35	14.10, 0.17	83.48, 0.11
10^5c	14.30	83.57		
10^4k	15.45	83.56		
10^4s	16.45	83.33	16.30, 0.78	83.44, 0.11
10^4c	17.00	83.42		
10^3k	19.00	83.50		
10^3s	17.45	83.39	18.87, 1.35	83.45, 0.06
10^3c	20.15	83.47		
100k	20.15	83.47		
100s	18.45	83.09	20.35, 2.00	83.33, 0.21
100c	22.45	83.42		
10k	22.30	83.52		
10s	21.00	83.42	22.43, 1.50	83.42, 0.1
10c	24.00	83.33		
1k	23.15	83.52		
1s	22.30	83.42	23.92, 2.11	83.41, 0.12
1c	26.30	83.28		
0.1k	36.00	83.41		
0.1s	-[d]	83.43	34.65, 1.91	83.39, 0.06
0.1c	33.30	83.33		

[#] The assay was performed in Genie III Real-time fluorometer, with the amplification times and annealing temperatures recorded at the end of each run. The samples were tested in different runs; *Template was serially diluted *C. pecorum* gDNA which genome copy number was determined by qPCR; from [a]: koala Marsbar isolate; [b]: sheep IPA isolate; and [c]: cattle E58 isolate; [d]: No amplification, but melt and annealing curve recorded.

Table 4(on next page)

Reproducibility of the LAMP testing using clinical and cultured samples.

^The assay was performed in Genie III Real-time fluorometer, with the amplification times and annealing temperatures recorded at the end of each run. The samples were tested in three different runs by two different operators.

1 **Table 4. Reproducibility of the LAMP testing using clinical and cultured samples.**

Samples	Run^	Time to amplify (min)	Melt (°C)	Time (Mean + SD)	Melt (Mean + SD)
***C. pecorum* positive samples**					
Koala rectal swab	1	20.15	83.44	20.53, 0.54	83.32, 0.16
	2	20.30	83.37		
	3	21.15	83.14		
Marsbar DNA	1	13.50	83.50	13.27, 0.20	83.55, 0.06
	2	13.15	83.52		
	3	13.15	83.62		
Koala A2 DNA	1	12.00	83.35	11.43, 0.51	83.41, 0.05
	2	11.00	83.45		
	3	11.30	83.43		
RI koala UGT swab	1	17.00	83.34	17.72, 0.62	83.21, 0.12
	2	18.00	83.21		
	3	18.15	83.09		
L14 DNA	1	13.15	83.53	13.15, 0	83.50, 0.02
	2	13.15	83.50		
	3	13.15	83.48		
HsLuRz DNA	1	13.45	83.49	13.63, 0.32	83.40, 0.08
	2	13.45	83.36		
	3	14.00	83.34		
K20 cloaca swab	1	22.00	82.83	22.2, 0.23	83.01, 0.19
	2	22.15	83.00		
	3	22.45	83.20		
***C. psittaci* positive samples**					
Cr009 DNA	1	6.45	84.30	6.40, 0.09	84.33, 0.03
	2	6.45	84.36		
	3	6.30	84.34		
HoRE DNA	1	5.00	84.46	5.10, 0.08	84.45, 0.14
	2	5.15	84.58		
	3	5.15	84.30		
B2 DNA	1	10.30	84.08	10.10, 0.17	84.17, 0.08
	2	10.00	84.20		
	3	10.00	84.24		
Horse placental swab	1	11.15	82.90	10.58, 0.49	83.19, 0.26
	2	10.30	83.42		
	3	10.30	83.24		
Horse_pl DNA	1	10.30	84.53	10.87, 0.98	84.41, 0.18
	2	12.00	84.21		
	3	10.30	84.50		

^The assay was performed in Genie III Real-time fluorometer, with the amplification times and annealing temperatures recorded at the end of each run. The samples were tested in three different runs by two different operators.

Table 5(on next page)

Comparison of the *C. psittaci* LAMP and qPCR methods for the organism detection in clinical samples.

Table 5. Comparison of the *C. psittaci* LAMP and qPCR methods for the organism detection in clinical samples.

Test	**qPCR +ve**	**qPCR -ve**	qPCR Total
LAMP +ve	11	1	12
LAMP -ve	1	13	14
LAMP Total	12	14	26

Table 6(on next page)

Comparison of the *C. pecorum* LAMP and qPCR methods for the organism detection in clinical samples.

Table 6. Comparison of the *C. pecorum* LAMP and qPCR methods for the organism detection in clinical samples.

Test	**16s +ve**	**16s -ve**	16s Total
LAMP +ve	29	7	36
LAMP -ve	3	24	27
LAMP Total	32	31	63

Table 7 (on next page)

Comparison of *C. pecorum* LAMP and qPCR for organism detection using rapidly processed swab samples and their DNA extracts.

Table 7. Comparison of *C. pecorum* LAMP and qPCR for organism detection using rapidly processed swab samples and their DNA extracts.

Sample	LAMP[a] result for swab suspension	qPCR[b] result for swab suspension	LAMP result for DNA extract	qPCR result for DNA extract	LAMP result for "spiked" swab suspension	LAMP result for "spiked" DNA extract	qPCR result for "spiked" swab suspension	qPCR result for "spiked" DNA extract
K1 ocular*	NEG	NEG	0.00/83.49	NEG	NEG	-	NEG	-
K6 ocular*	NEG	NEG	21.00/83.23	3x10^3 (Ct 20)	NEG	-	NEG	-
K9 ocular*	NEG	NEG	25.45/83.39	287 (Ct 24)	NEG	-	NEG	-
K2 ocular*	NEG	NEG	NEG	NEG	NEG	-	NEG	-
R1 eye	25.45/83.39	222 (Ct 25)	20.15/83.27	750 (Ct 24)	-	-	-	-
R1 cloaca	30.00/83.34	NEG	NEG	NEG	11.15/83.47	12.15/83.42	5x10^3 (Ct 17)	1.5x10^3 (Ct 18)
K eye	27.00/83.15	NEG	0.00/83.35	NEG	-	-	-	-
Koala 2 eye	NEG	NEG	NEG	NEG	11.00/83.51	11.00/83.40	1.2x10^3 (Ct 19)	1.1x10^4 (Ct 15)
Koala 2 cloaca	27.30/83.77	116 (Ct 26)	21.30/83.49	375 (Ct 25)	-	-	-	-
Will Cloaca	0.00/83.77	NEG	NEG	NEG	12.00/83.45	11.00/83.34	1.5x10^3 (Ct 19)	8x10^3 (Ct 17)
23117 Eye	21.30/83.20	NEG	23.15/83.23	150 (Ct 25)	-	-	-	-
23117 Cloaca	22.00/83.29	NEG	24.00/83.15	90 (Ct 27)	-	-	-	-
Flyn eye	NEG	NEG	NEG	NEG	12.30/83.50	11.00/83.35	1.9x10^3 (Ct 18)	8.3x10^3 (Ct 16)
Tyke eye	NEG	NEG	NEG	NEG	12.00/83.44	10.45/83.40	1.3x10^3 (Ct 19)	9x10^3 (Ct 16)
Bill eye	NEG	NEG	NEG	NEG	12.15/83.49	10.45/83.34	1.2x10^3 (Ct 19)	1x10^4 (Ct 15)
Ray eye	NEG	NEG	NEG	NEG	12.45/83.49	11.00/83.40	4.7x10^3 (Ct 17)	1x10^4 (Ct 15)
Ray cloaca	NEG	NEG	NEG	NEG	12.15/83.43	11.00/83.30	700 (Ct 20)	9x10^3 (Ct 16)
Koala F Eye	NEG	NEG	NEG	NEG	11.45/83.45	11.00/83.35	1.3x10^3 (Ct 19)	1.1x10^4 (Ct 15)

[a]: LAMP results are expressed as time to amplify (min) and melt (°C); [b]: qPCR results are expressed as copies/reaction and Ct value; *: RNA Later swabs.