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

Martina Jelocnik[a#], Md Mominul Islam[a], Danielle Madden[a], Cheryl Jenkins[b], James Branley[c], Scott Carver[d], Adam Polkinghorne[a]

[a] Centre for Animal Health Innovation, University of the Sunshine Coast, Maroochydore, QLD 4558, Australia.

[b] NSW Department of Primary Industries, Elizabeth Macarthur Agricultural Institute, Menangle, NSW 2568, Australia.

[c] Nepean Hospital, Penrith, NSW 2751, Australia.

[d] School of Biological Sciences, University of Tasmania, Hobart TAS 7001, Australia.

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

#Corresponding author: Martina Jelocnik, martina.jelocnik@research.usc.edu.au

Abstract

Background

Chlamydia psittaci and *Chlamydia pecorum* are important veterinary pathogens, with the former also being responsible for zoonoses, and the later 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 263bp region of the *C. psittaci*-specific Cps_0607 gene or a 209bp 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 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. 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 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, manifesting as asymptomatic shedding to acute respiratory disease and, more recently, 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 can be asymptomatic or can manifest as 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

103 considering most chlamydial infections of veterinary concern, the ability to provide a rapid
104 detection of infections becomes of increasing significance when veterinarians and other animal
105 workers may be at risk of being exposed to *C. psittaci* infections in field or farm settings. POC
106 testing is also particularly attractive for *Chlamydia* detection in wild animals due to the typical
107 logistics associated with field sampling and treatment or because no other routine testing is
108 available. The latter problem is particularly acute for diagnosing infections in koalas, with the
109 recent decision to stop the production of a commercially viable solid-phase ELISA leaving
110 wildlife hospitals unable to diagnose and successfully treat asymptomatic *C. pecorum* infections
111 (Hanger *et al.* 2013).

112

113 While there are many options for molecular POC diagnostics, Loop Mediated Isothermal
114 Amplification (LAMP) assays developed for use in pathogen diagnostics are popular as they
115 offer significant advantages over PCR and/or serology testing (Maffert *et al.* 2017). Rapid,
116 simple, highly specific, easy to interpret, and carried out at a constant temperature, LAMP assays
117 can provide a diagnosis in 30 min, in either laboratory or field setting (Mansour *et al.* 2015;
118 Notomi *et al.* 2015). Rapid isothermal LAMP assays that could be performed at the POC
119 targeting human *C. pneumoniae* (Kawai *et al.* 2009) and *C. trachomatis* (Jevtusevskaja *et al.*
120 2016; Choopara *et al.* 2017) infections have been proposed for use in chlamydial diagnostics.
121 Development of a *C. pecorum* LAMP, in particular, would meet immediate demand for koala *C.*
122 *pecorum* infections diagnostics, providing an alternative solution for the current laboratory
123 diagnostics. Recent Australian outbreak of psittacosis in veterinary staff and students, involved
124 in treating a *C. psittaci*-infected and sick neonatal foal (Chan *et al.* 2017; Jelocnik *et al.* 2017),
125 further demonstrates the need for POC assays such as LAMP to rapidly diagnose *C. psittaci*. In

126 the present study, we describe the development and evaluation of rapid and robust *C. psittaci*-
127 specific and *C. pecorum*-specific LAMP assays for detection of these organisms in either
128 laboratory or POC settings.

129

130 **Materials and methods**

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

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

141 *C. pecorum* LAMP was evaluated using a: 1) 18 DNA samples extracted from previously
142 characterised seven koala, four sheep, four cattle and three pig *C. pecorum* cultures (Table S1);
143 2) 16 sheep and 13 cattle ocular, rectal, and tissue swab DNA samples; and 3) 34 ocular and
144 UGT koala swab DNA samples (Table S3), all available in our collection. The use of these
145 swabs, also collected by qualified veterinarians as a part of routine diagnostic testing, was
146 considered by the University of The Sunshine Coast (USC) Animal Ethics Committee, and is
147 under Animal ethics approval exemptions (AN/E/14/01 and AN/E/14/31).

148

149 We also evaluated specificity of the assays against DNA samples extracted from previously
150 characterised: 1) related chlamydial isolates (koala C. *pneumoniae* LPColN, *C. abortus* S26/3, *C.*
151 *suis* S45, *C. trachomatis* serovar D, *C. murridarum* Nigg, *C. caviae* GPIC) and uncultured
152 *Chlamydiales* (*Fritschea* spp.); 2) Gram negative *Escherichia coli* and *Prevotella bivia*; Gram
153 positive *Fusobacterium nucleatum*, *Staphylococcus epidermidis*, *S. aureus*, *Streptococcus* spp.,
154 and *Enterococcus faecalis*; and 3) commercially available human gDNA (Promega, Alexandria,
155 NSW 2015), all available in our laboratory (Table S1).

156

157 In order to evaluate rapid swab processing, 12 ocular, cloacal and UGT (eight dry and four RNA-
158 Later) clinical swabs taken from nine koalas with presumptive chlamydiosis were used for
159 testing without DNA extraction. Briefly, RNA-Later and dry swabs with added 500ul TE buffer
160 were vortexed vigoursly for 5 min. 300ul aliquots were then heated to 98°C for 15 min to lyse
161 DNA, following LAMP testing. The use of these swabs, collected as a part of routine diagnostic
162 testing, is also under Animal ethics approval exemption (AN/E/14/01).

163

164 *LAMP assays design*

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

170 The *C. pecorum*-specific candidate gene, encoding for a single-copy conserved hypothetical
171 protein and denoted CpecG_0573 in the representative *C. pecorum* MC/Marsbar strain (Genbank
172 accession number NZ_CM002310.1), was determined from the ongoing comparative genomics
173 using published and newly sequenced 50 koala and livestock *C. pecorum* genomes, available in
174 our database. For the purposes of this study, we will refer to it as *Cpec*_HP. Both candidate gene
175 sequences were aligned to the corresponding allele from other publicly available *C. psittaci* or *C.*
176 *pecorum* strains using Clustal X (as implemented in Geneious 9 (Kearse *et al.* 2012)), and
177 analysed in BLASTn to assess intra-species sequence identity, and inter-species specificity.

178

179 For *C. ps*_0607 alignment, besides 6BC, we used this gene alleles from strains 84/55
180 (CP003790.1), 02DC15 (CP002806.1), 01DC11 (CP002805.1), WC (CP003796.1), 01DC12
181 (HF545614.1), NJ1 (CP003798.1), CR009 (LZRX01000000), Ho Re upper (LZRE01000000)
182 and PoAn (LZRG01000000). For *C. pec*_HP alignment, besides MC/Marsbar, we used this
183 gene alleles from E58 (CP002608.1), P787 (CP004035.1), W73 (CP004034.1), IPA
184 (NZ_CM002311.1), NSW/Bov/SBE (NZ_JWHE00000000.1), L71 (LFRL01000000), L17
185 (LFRK01000001), L1 (LFRH00000000), DBDeUG (NZ_CM002308.1), SA/K2/UGT
186 (SRR1693792), Nar/S22/Rec (SRR1693794) and Mer/Ovi1/Jnt (SRR1693791).

187

188 Species-specific LAMP primers were designed using the target sequences with the open-source
189 Primer Explorer v5 software (Eiken Chemical Co., Japan) and licensed LAMP Designer 1.15
190 software (Premier Biosoft, USA). For both *C. pecorum* and *C. psittaci*, Primer Explorer v5
191 yielded five sets of four LAMP primers including two outer (forward F3 and backward B3)

primers and two inner (forward inner FIP and backward inner BIP) primers targeting different regions of the target gene, while LAMP Designer yielded single best set of six LAMP primers including two outer primers (forward F3 and backward B3), two inner primers (forward inner FIP and backward inner BIP) and two loop primers (forward loop LF and backwards loop LB).

After *in silico* and in LAMP reaction testing, a set of four primers designed by PrimerExplorer v5 and targeting 209bp region of the *C. pec*_HP gene (spanning from position 22 to 230) was selected for *C. pecorum* LAMP assays performed in this study. Additional loop primers (LF/LB) were also designed to speed up amplification time and increase sensitivity. For *C. psittaci*, a set of six primers designed with LAMP Designer and targeting 263bp region of the *C. ps*_0607 gene (spanning from position 286 to 548) was selected for LAMP assays performed in this study. All primer sequences specificity was evaluated using discontiquousBLAST analyses. Amplicons generated by conventional PCR using outer F3 and B3 primers for both *C. psittaci* 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 same temperature and annealing conditions, however for 45 min. A negative control (LAMP mix only) was included in each run. We also run several *C. pecorum* LAMP assays using four primer set, two outer (F3 and B3) and two inner (FIP and BIP) primers, on a thermal cycle heating block at 65°C for 45 min, following detection of amplicons by electrophoresis on a 1.5% ethidium bromide agarose gel and visualisation under UV.

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 *et al.* 2011), while *C. psittaci*-presumptive samples were tested using *Pan-Chlamydiales* qPCR assay targeting 16S rRNA, following amplicon sequencing to confirm species identity (Everett, Bush & Andersen 1999). The qPCR assays were carried out in a 20 µL total volume, consisting of 10 µL SYBR™ Green PCR Master Mix (Life Technologies Australia Pty Ltd., Scoresby, Vic 3179), 1 µL of each 10 µM forward and reverse primer, 3 µL miliqH2O, and 5 µL DNA template. The qPCR assays were run for 35 cycles (Ct), and in each qPCR assay a positive (cultured *C. pecorum* and/or *C. psittaci* DNA) and negative (miliqH2O) controls were included. Based on the qPCR standard curve and the number of running cycles, samples amplifying at > 30 Ct (and/or equivalent detected genome copy number) were considered negative. The 23 *C. psittaci*-presumptive equine samples were also tested with C. *psittaci*-specific qPCR assay targeting the 16S rRNA gene/16S-23S rRNA spacer gene (Madico *et al.* 2000) at the State

Veterinary Diagnostic Laboratory (SVDL), Elizabeth Macarthur Agricultural Institute (EMAI), Menangle, NSW, Australia. Samples amplifying at > 39 Ct were considered negative. LAMP testing was performed in a blind fashion, by two different operators, unaware of qPCR results.

Statistical analyses

For each assay, we compared the performance of two tests evaluated in the same population by calculating Kappa and overall agreement, as well as estimated sensitivity and specificity (with specified Clopper-Pearson (exact) confidence limits) of LAMP compared to the known reference (gold standard) qPCR test using EpiTools online (Sergeant 2017).

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.

258 *C. psittaci* and *C. pecorum* LAMP development

259 A *C. psittaci*-specific gene (*C.ps*_0607) was previously characterised as a conserved gene
260 sequence present only in *C. psittaci* genomes, and absent from all other related chlamydial
261 species (Voigt *et al.* 2012). BLAST analyses and alignment of the *C.ps*_0607 gene sequences,
262 including those from recently described human, bird and equine Australian isolates, confirmed
263 species specificity and sequence conservation. Between 0 and 13 single nucleotide
264 polymorphisms (SNPs) were observed amongst strains (100-95.1% sequence identity) based on a
265 263 bp alignment of *C.ps*_0607 gene sequences, including that from the most distant *C. psittaci*
266 NJ1 taxon (Figure S1A). Similarly, the *C. pecorum* HP gene (denoted CpecG_0573 locus in
267 Marsbar strain) was determined as a highly conserved species-specific sequence following
268 BLAST analysis against publicly available sequences. Using an alignment of HP gene sequences
269 from 14 publicly available *C. pecorum* genomes, there were only 2 SNPs in the 209bp region to
270 be targeted by LAMP (Figure S1B).

271

272 Although multiple LAMP primer sets were predicted, LAMP primer sets denoted in Figure 1
273 were chosen for further assay development. For *C. psittaci* assays, a set designed using LAMP
274 Explorer was utilised; while for *C. pecorum*, we used a set designed with PrimerExplorer (Table
275 1). After initial testing, some of the predicted primer sets were discarded due to: 1) potential
276 cross-amplification and non-specificity of their sequences; 2) not achieving amplification signal
277 in the fluorometer; and 3) amplifying non-specific targets, including positive amplification in
278 negative controls (data not shown). While we achieved initial amplification of a *C. psittaci* single
279 copy dilution in a 30 min assay using the designed LAMP primer set, initial reaction times for a
280 *C. pecorum* single copy amplification averaged 50 min. In order to speed up amplification times

281 for *C. pecorum*, we additionally designed a pair of Loop primers for the *C. pecorum* set which
282 decreased the amplification of a single copy to 30 min.

283

284 *In silico* species-specificity for *C. psittaci* and *C. pecorum* LAMP targets was tested in the
285 developed LAMP assays. Positive amplification (an observable amplification curve characterised
286 by a specific melt) was observed for the target species only, as evaluated using 12 *C. psittaci* and
287 18 *C. pecorum* cultured isolates, DNA from related chlamydial isolates and a range of DNA
288 extracts from other bacteria (Table S1). The *C. pecorum* and *C. psittaci* LAMP assays did not
289 amplify either the related chlamydial species or other bacteria included in our specificity assays
290 (Table S1). In this study, in contrast, a previously described "*C. pecorum*-specific" qPCR assay
291 (Marsh *et al.* 2011; Wan *et al.* 2011) showed positive amplification and melt for *C. psittaci* and
292 *C. pneumoniae* DNA samples.

293 With species-specificity previously confirmed for the *C. ps*_0607 gene by comparative genomics
294 and in diagnostic assays (Voigt *et al.* 2012; Opota *et al.* 2015), the choice to use this gene as a
295 LAMP target was straightforward. For *C. pecorum*, however, we utilised our ongoing
296 comparative genomics to select *C. pecorum*-specific and conserved *C.pec*_HP gene described in
297 this study for the first time. *In silico* analyses and assay development confirmed species-
298 specificity of this gene and its suitability for use in diagnostic assays. Previously published *C.*
299 *pecorum* diagnostic assays targeted highly polymorphic genes such as *omp*A (Higgins *et al.*
300 2012; Yang *et al.* 2014), which may require the use of probes due to sequence variation,
301 prolonging the detection time and increasing diagnostic costs. Our routinely used ~~*in house*~~ *C.*
302 *pecorum*-specific assay which targets a 204 bp 16S rRNA fragment (Marsh *et al.* 2011; Wan *et*
303 *al.* 2011) was simpler to use, however we have shown that this assay may cross-react with other

304 related chlamydial species due to recognised sequence conservation and synteny characteristic
305 for chlamydial organisms (Bachmann, Polkinghorne & Timms 2014). For koala diagnostics
306 where *C. pecorum* is the most abundant and prevalent chlamydial organism (Polkinghorne *et al.*
307 2013), this cross-reactivity may not be of a big concern. For the veterinary diagnosis of
308 infections in livestock where co-infections with several chlamydial species are common (Lenzko
309 *et al.* 2011; Reinhold *et al.* 2011), this assay may not be suitable for use. Using *C. pecorum*-
310 specific HP gene as a target in different diagnostic assays would hence seem promising.

311

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

313 The sensitivity of the LAMP assays was evaluated using 5µL cultured *C. psittaci* and *C.*
314 *pecorum* gDNA in 10-fold serial dilutions as a template in assays performed in triplicate in
315 separate runs. The limits of detection of the LAMP assays were conservatively 10 copies for *C.*
316 *psittaci*, with 3/3 (100%) positive amplification for 10 copies dilution for *C. psittaci*, and 1 copy
317 for *C. pecorum*, with 3/3 (100%) positive amplification for a single copy dilution for *C. pecorum*
318 (Table 2 and 3). In the final and optimised LAMP assays, the mean amplification time detecting
319 the lower limit (a single copy) for *C. psittaci* was 14.23 min with an average 84.45°C melt
320 (Table 2) while, for *C. pecorum* it was 24 min with an average 83.42°C melt (Table 3).
321 Comparing the two newly developed assays, *C. psittaci* LAMP had the faster run time than that
322 of *C. pecorum* LAMP. This difference in assays kinetics could be attributed to the improved *C.*
323 *psittaci* LAMP primers design, as they were predicted by the LAMP Designer software
324 (Nagamine, Hase & Notomi 2002). As we designed additionally Loop primers for *C. pecorum*,
325 we can anticipate an improvement in the 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 with high to low infectious loads (Table 4). All samples were run in a "blind fashion", in triplicate and in separate runs by two different operators. For both assays, the amplification times and melts of each sample between the runs were very similar, with 0 to 1.5 min (SDs ranging from 0 – 0.98) difference in amplification times for each sample, and 0.03 to 0.83°C (SDs ranging from 0.02 – 0.26) difference in melt for each sample. Congruence between the runs performed by different operators indicates that both LAMP assays described in this study are highly reproducible, and can detect the target organism in less than 30 min even when in low infectious loads of < 10 copies.

Pathogen detection in clinical samples using newly developed LAMP

For *C. psittaci*, a total of 27 DNA extracts from clinical samples were tested with both *C. psittaci* LAMP and qPCR assays (Table S2). For these analyses, samples with >20 min amplification time were considered negative for LAMP, while for qPCR, samples with <20 genome copy/reaction and/or > 30 Ct (quantification cycle) were considered negative, based on the qPCR standard curve and the number of running cycles used for this testing. As observed in Table S2 and based on above cut-off values, 24/26 (92.3%) samples were congruent between the two tests, with 11 samples positive and 13 samples negative by both (Table 5). For 2/26 (7.7%) where there was disagreement, one sample was LAMP positive but qPCR negative, and another was

348 qPCR positive but *C. psittaci* LAMP negative. Based on these results, the Kappa value was
349 calculated at 0.85 (95% CI 0.64 – 1.05) indicating an almost perfect agreement between the tests.
350 The overall sensitivity of the *C. psittaci* LAMP was 91.7% (Clopper-Pearson 95% CI 0.62 –
351 0.99) and with 92.9% (Clopper-Pearson 95% CI 0.66 – 0.99) specificity, compared to the qPCR
352 used in this study. In addition, a subset of 23 samples was also tested independently by a third
353 party. Using a cut off of > Ct 39 as negative, 19/23 (82.60 %) of these test results were in
354 congruence with our *C. psittaci* LAMP results (Table S2).

355

356 For *C. pecorum*, we tested a total of 63 DNA extracts from clinical samples from several animal
357 hosts by both LAMP and qPCR (Table S3). For these analyses, samples with > 30 min
358 amplification time were considered negative for LAMP, while for qPCR, samples with < 35
359 genome copy /reaction and/or > 30 Ct were considered negative based on the standard curve and
360 number of run cycles used for this testing. For the 63 clinical samples, the overall congruence
361 was 84.1% with a Kappa value of 0.68 (95% CI 0.50 – 0.86), indicating substantial agreement
362 between the tests. Congruent results between tests were obtained for 53 samples, while there
363 were 10 discrepant samples using the above cut off for *C. pecorum* (Table 6). The overall
364 sensitivity of *C. pecorum* LAMP was 90.6 % (Clopper-Pearson 95% CI 0.75 – 0.98), while
365 specificity was 77.4 % (Clopper-Pearson 95% CI 0.59 – 0.90) in comparison to the qPCR assay.
366 A subset of 36 *C. pecorum* samples was also tested in a thermal cycler using newly developed
367 LAMP and results were determined as an end point detection. For this experiment, 34/36
368 (94.4%) samples were congruent between LAMP performed in fluorometer and in thermal cycler
369 (Table S3), demonstrating robustness of the *C. pecorum* LAMP (Figure S2).

370

Considering that the qPCR assay used in this study to quantify and detect *C. psittaci* is chlamydial genus rather species specific (Everett *et al.* 1999), high congruence observed for *C. psittaci* assays could be attributed to testing a limited set of samples taken from hosts with 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. Both targets seem suitable for use in preliminary 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 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 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 12 swabs taken from conjunctival and urogenital sites from koalas with presumptive chlamydiosis, of which four were stored in RNA Later and eight 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

394 performed in fluorometer and qPCR assay. We also performed DNA extraction from the four
395 RNA Later swabs to be used as a comparison to rapid swab processing. We did not detect *C.*
396 *pecorum* DNA in any of the RNA Later suspensions either by LAMP nor qPCR assay (Table 7),
397 in contrast to detecting *C. pecorum* in 50% (2/4) of the DNA extracts from the swabs by both
398 methods. *C. pecorum* was detected in 6/8 rapidly processed dry swabs samples by LAMP, and
399 only 2/8 by qPCR (Table 7). Our results suggest that the LAMP assays are capable of amplifying
400 specific amplification products from crude DNA extracts. We did not perform or evaluate the
401 potential presence of inhibitors using these approaches but this would be a logical step in the
402 further evaluation of these assays.

403

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

413

414 **Acknowledgments**

We thank Prof. James Mahoney, Dr. Ian Marsh, Narelle Sales and Dr. Bill Lott for their helpful advice on POC assays. We also thank Dr. Brendon O'Rourke, Sankhya Bommana, Sharon Nyari, Noa Ziklo and Alyce Taylor-Brown for provision of DNA samples used in this study.

418 **References**

419 Bachmann NL, Polkinghorne A, Timms P. 2014. Chlamydia genomics: providing novel insights
420 into chlamydial biology. *Trends in Microbiology* 22:464-472.
421 DOI:10.1016/j.tim.2014.04.013
422 Branley J, Bachmann NL, Jelocnik M, Myers GS, Polkinghorne A. 2016. Australian human and
423 parrot *Chlamydia psittaci* strains cluster within the highly virulent 6BC clade of this
424 important zoonotic pathogen. *Scientific Reports* 6:30019. DOI:10.1038/srep30019
425 Branley JM, Roy B, Dwyer DE, Sorrell TC. 2008. Real-time PCR detection and quantitation of
426 *Chlamydophila psittaci* in human and avian specimens from a veterinary clinic cluster.
427 *European Journal of Clinical Microbiology and Infectious Diseases* 27:269-273.
428 DOI:10.1007/s10096-007-0431-0
429 Branley JM, Weston KM, England J, Dwyer DE, Sorrell TC. 2014. Clinical features of endemic
430 community-acquired psittacosis. *New Microbes New Infections* 2:7-12.
431 DOI:10.1002/2052-2975.29
432 Burnard D, and Polkinghorne A. 2016. Chlamydial infections in wildlife–conservation threats
433 and/or reservoirs of 'spill-over' infections? *Veterinary Microbiology* 196:78-84.
434 http://dx.doi.org/10.1016/j.vetmic.2016.10.018
435 Bustin SA, Beaulieu JF, Huggett J, Jaggi R, Kibenge FS, Olsvik PA, Penning LC, Toegel S.
436 2010. MIQE precis: Practical implementation of minimum standard guidelines for
437 fluorescence-based quantitative real-time PCR experiments. *BMC Molecular Biology*
438 11:74. DOI:10.1186/1471-2199-11-74
439 Chan J, Doyle B, Branley J, Sheppeard V, Gabor M, Viney K, Quinn H, Janover O, McCready
440 M, Heller J. 2017. An outbreak of psittacosis at a veterinary school demonstrating a novel
441 source of infection. *One Health* 3:29-33.
442 Choopara I, Arunrut N, Kiatpathomchai W, Dean D, Somboonna N. 2017. Rapid and visual
443 *Chlamydia trachomatis* detection using loop-mediated isothermal amplification and
444 hydroxynaphthol blue. *Letters in Applied Microbiology* 64:51-56.
445 DOI:10.1111/lam.12675
446 Everett KDE, Bush RM, Andersen AA. 1999. Emended description of the order *Chlamydiales*,
447 proposal of *Parachlamydiaceae* fam. nov. and *Simkaniaceae* fam. nov., each containing
448 one monotypic genus, revised taxonomy of the family *Chlamydiaceae*, including a new

449 genus and five new species, and standards for the identification of organisms.
450 *International Journal of Systematic Bacteriology* 49:415-440. DOI:10.1099/00207713-
451 49-2-415
452 Gaede W, Reckling KF, Dresenkamp B, Kenklies S, Schubert E, Noack U, Irmscher HM,
453 Ludwig C, Hotzel H, Sachse K. 2008. *Chlamydophila psittaci* infections in humans
454 during an outbreak of psittacosis from poultry in Germany. *Zoonoses Public Health*
455 55:184-188. DOI:10.1111/j.1863-2378.2008.01108.x
456 Geens T, Dewitte A, Boon N, Vanrompay D. 2005. Development of a *Chlamydophila psittaci*
457 species-specific and genotype-specific real-time PCR. *Veterinary Research* 36:787-797.
458 Gonzalez-Astudillo V, Allavena R, McKinnon A, Larkin R, Henning J. 2017. Decline causes of
459 Koalas in South East Queensland, Australia: a 17-year retrospective study of mortality
460 and morbidity. *Scientific Reports* 7:42587. DOI:10.1038/srep42587
461 Hanger J, Loader J, Wan C, Beagley KW, Timms P, Polkinghorne A. 2013. Comparison of
462 antigen detection and quantitative PCR in the detection of chlamydial infection in koalas
463 (*Phascolarctos cinereus*). *The Veterinary Journal* 195:391-393.
464 http://dx.doi.org/10.1016/j.tvjl.2012.07.024
465 Higgins DP, Beninati T, Meek M, Irish J, Griffith JE. 2012. Within-population diversity of koala
466 *Chlamydophila pecorum* at *omp*A VD1-VD3 and the ORF663 hypothetical gene.
467 *Veterinary Microbiology* 156:353-358. 10.1016/j.vetmic.2011.11.005
468 Jelocnik M, Bachmann NL, Kaltenboeck B, Waugh C, Woolford L, Speight KN, Gillett A,
469 Higgins DP, Flanagan C, Myers GS, Timms P, Polkinghorne A. 2015a. Genetic diversity
470 in the plasticity zone and the presence of the chlamydial plasmid differentiates
471 *Chlamydia pecorum* strains from pigs, sheep, cattle, and koalas. *BMC Genomics* 16:893.
472 DOI:10.1186/s12864-015-2053-8
473 Jelocnik M, Branley J, Heller J, Raidal S, Alderson S, Galea F, Gabor M, Polkinghorne A. 2017.
474 Multilocus sequence typing identifies an avian-like *Chlamydia psittaci* strain involved in
475 equine placentitis and associated with subsequent human psittacosis. *Emerging Microbes*
476 *and Infection* 6:e7. DOI:10.1038/emi.2016.135
477 Jelocnik M, Self R, Timms P, Borel N, Polkinghorne A. 2015b. Novel sequence types of
478 *Chlamydia pecorum* infect free-ranging Alpine ibex (*Capra ibex*) and red deer (*Cervus*

elaphus) in Switzerland. *Journal of Wildlife Diseases* 51:479-483. DOI:10.7589/2014-08-220

Jevtusevskaja J, Uusna J, Andresen L, Krolov K, Laanpere M, Grellier T, Tulp I, Langel U. 2016. Combination with antimicrobial peptide lyses improves loop-mediated isothermal amplification based method for *Chlamydia trachomatis* detection directly in urine sample. *BMC Infectious Diseases* 16:329. DOI:10.1186/s12879-016-1674-0

Kawai Y, Miyashita N, Kishi F, Tabuchi M, Oda K, Yamaguchi T, Kawasaki K, Yamazaki T, Ouchi K. 2009. Development and evaluation of a loop-mediated isothermal amplification method for the rapid detection of *Chlamydophila pneumoniae*. *European Journal of Clinical Microbiology and Infectious Diseases* 28:801-805. DOI:10.1007/s10096-009-0710-z

Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Meintjes P, Drummond A. 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* 28:1647-1649. DOI:10.1093/bioinformatics/bts199

Knittler MR, and Sachse K. 2015. *Chlamydia psittaci*: update on an underestimated zoonotic agent. *Pathogens and Disease* 73:1-15. DOI:10.1093/femspd/ftu007

Laroucau K, Aaziz R, Meurice L, Servas V, Chossat I, Royer H, de Barbeyrac B, Vaillant V, Moyen JL, Meziani F, Sachse K, Rolland P. 2015. Outbreak of psittacosis in a group of women exposed to *Chlamydia psittaci*-infected chickens. *Euro Surveillance* 20:pii=21155.

Lenzko H, Moog U, Henning K, Lederbach R, Diller R, Menge C, Sachse K, Sprague L. 2011. High frequency of chlamydial co-infections in clinically healthy sheep flocks. *BMC Veterinary Research* 7:29. DOI:10.1186/1746-6148-7-29

Madico G, Quinn TC, Boman J, Gaydos CA. 2000. Touchdown enzyme time release-PCR for detection and identification of *Chlamydia trachomatis*, *C. pneumoniae*, and *C. psittaci* using the 16S and 16S-23S spacer rRNA genes. *Journal of Clinical Microbiology* 38:1085-1093.

508 Maffert P, Reverchon S, Nasser W, Rozand C, and Abaibou H. 2017. New nucleic acid testing
509 devices to diagnose infectious diseases in resource-limited settings. *European Journal of*
510 *Clinical Microbiology and Infectious Diseases*. DOI:10.1007/s10096-017-3013-9
511 Mahony J, Chong S, Bulir D, Ruyter A, Mwawasi K, Waltho D. 2013. Development of a
512 sensitive loop-mediated isothermal amplification assay that provides specimen-to-result
513 diagnosis of respiratory syncytial virus infection in 30 minutes. *Journal of Clinical*
514 *Microbiology* 51:2696-2701. DOI:10.1128/jcm.00662-13
515 Mansour SM, Ali H, Chase CC, and Cepica A. 2015. Loop-mediated isothermal amplification
516 for diagnosis of 18 World Organization for Animal Health (OIE) notifiable viral diseases
517 of ruminants, swine and poultry. *Animal Health Research Reviews* 16:89-106.
518 DOI:10.1017/s1466252315000018
519 Marsh J, Kollipara A, Timms P, Polkinghorne A. 2011. Novel molecular markers of *Chlamydia*
520 *pecorum* genetic diversity in the koala (*Phascolarctos cinereus*). *BMC Microbiology*
521 11:77. DOI:10.1186/1471-2180-11-77
522 McKenna JP, Cox C, Fairley DJ, Burke R, Shields MD, Watt A, Coyle PV. 2017. Loop-
523 mediated isothermal amplification assay for rapid detection of *Streptococcus agalactiae*
524 (group B streptococcus) in vaginal swabs - a proof of concept study. *Journal of Medical*
525 *Microbiology* 66:294-300. DOI:10.1099/jmm.0.000437
526 Menard A, Clerc M, Subtil A, Megraud F, Bebear C, de Barbeyrac B. 2006. Development of a
527 real-time PCR for the detection of *Chlamydia psittaci*. *Journal of Medical Microbiology*
528 55:471-473. DOI:10.1099/jmm.0.46335-0
529 Nagamine K, Hase T, Notomi T. 2002. Accelerated reaction by loop-mediated isothermal
530 amplification using loop primers. *Molecular and Cellular Probes* 16:223-229.
531 Notomi T, Mori Y, Tomita N, Kanda H. 2015. Loop-mediated isothermal amplification (LAMP):
532 principle, features, and future prospects. *Journal of Microbiology* 53:1-5.
533 DOI:10.1007/s12275-015-4656-9
534 Opota O, Jaton K, Branley J, Vanrompay D, Erard V, Borel N, Longbottom D, Greub G. 2015.
535 Improving the molecular diagnosis of *Chlamydia psittaci* and *Chlamydia abortus*
536 infection with a species-specific duplex real-time PCR. *Journal of Medical Microbiology*
537 64:1174-1185. DOI:10.1099/jmm.0.000139

538 Parida M, Sannarangaiah S, Dash PK, Rao PV, Morita K. 2008. Loop mediated isothermal
539 amplification (LAMP): a new generation of innovative gene amplification technique;
540 perspectives in clinical diagnosis of infectious diseases. *Reviews in Medical Virology*
541 18:407-421. DOI:10.1002/rmv.593
542 Polkinghorne A, Hanger J, Timms P. 2013. Recent advances in understanding the biology,
543 epidemiology and control of chlamydial infections in koalas. *Veterinary Microbiology*
544 165:214-223. DOI:10.1016/j.vetmic.2013.02.026
545 Reinhold P, Sachse K, Kaltenboeck B. 2011. *Chlamydiaceae* in cattle: Commensals, trigger
546 organisms, or pathogens? *The Veterinary Journal* 189:257-267.
547 DOI:10.1016/j.tvjl.2010.09.003
548 Sachse K, Vretou E, Livingstone M, Borel N, Pospischil A, Longbottom D. 2009. Recent
549 developments in the laboratory diagnosis of chlamydial infections. *Veterinary*
550 *Microbiology* 135:2-21. http://dx.doi.org/10.1016/j.vetmic.2008.09.040
551 Sergeant ESG. 2017. Epitools epidemiological calculators. *Available at*
552 *http://epitools.ausvet.com.au*
553 Szymanska-Czerwinska M, and Niemczuk K. 2016. Avian Chlamydiosis zoonotic disease.
554 *Vector Borne and Zoonotic Diseases* 16:1-3. DOI:10.1089/vbz.2015.1839
555 Taylor-Brown A, and Polkinghorne A. 2017. New and emerging chlamydial infections of
556 creatures great and small. *New Microbes New Infections* 18:28-33.
557 DOI:10.1016/j.nmni.2017.04.004
558 Tomita N, Mori Y, Kanda H, Notomi T. 2008. Loop-mediated isothermal amplification (LAMP)
559 of gene sequences and simple visual detection of products. *Nature Protocols* 3:877-882.
560 DOI:10.1038/nprot.2008.57
561 Voigt A, Schöfl G, Saluz HP. 2012. The *Chlamydia psittaci* Genome: A comparative analysis of
562 intracellular pathogens. *PLoS ONE* 7:e35097. DOI:10.1371/journal.pone.0035097
563 Walker E, Lee EJ, Timms P, Polkinghorne A. 2015. *Chlamydia pecorum* infections in sheep and
564 cattle: A common and under-recognised infectious disease with significant impact on
565 animal health. *Veterinary Journal* 206:252-260. DOI:10.1016/j.tvjl.2015.09.022
566 Walker E, Moore C, Shearer P, Jelocnik M, Bommana S, Timms P, Polkinghorne A. 2016.
567 Clinical, diagnostic and pathologic features of presumptive cases of *Chlamydia pecorum*-

associated arthritis in Australian sheep flocks. *BMC Veterinary Research* 12:193. DOI:10.1186/s12917-016-0832-3

Wan C, Loader J, Hanger J, Beagley KW, Timms P, Polkinghorne A. 2011. Using quantitative polymerase chain reaction to correlate *Chlamydia pecorum* infectious load with ocular, urinary and reproductive tract disease in the koala (*Phascolarctos cinereus*). *Australian Veterinary Journal* 89:409-412. DOI:10.1111/j.1751-0813.2011.00827.x

Yang R, Jacobson C, Gardner G, Carmichael I, Campbell AJ, Ryan U. 2014. Longitudinal prevalence and faecal shedding of *Chlamydia pecorum* in sheep. *Veterinary Journal* 201:322-326. DOI:10.1016/j.tvjl.2014.05.037

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.

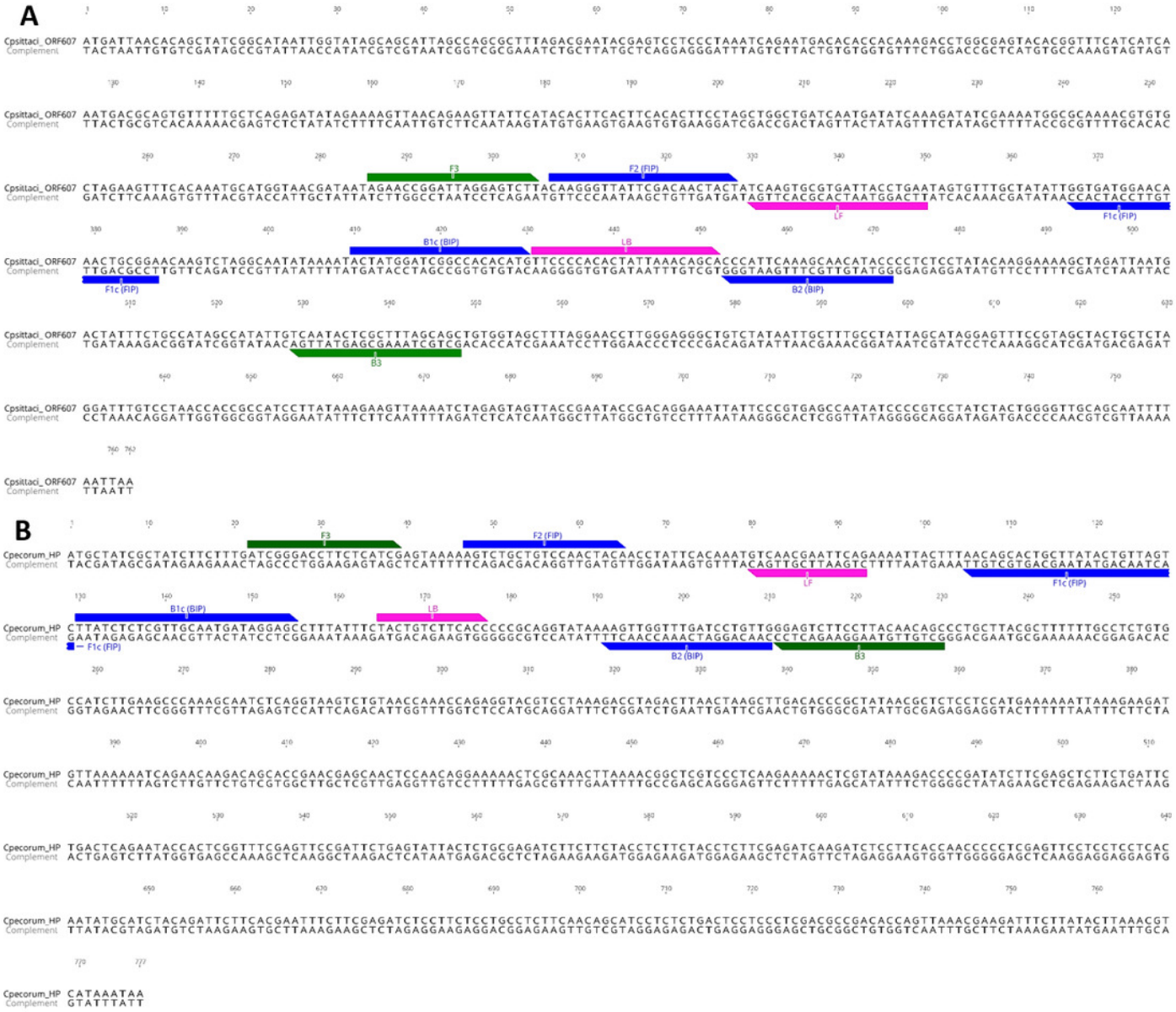

Table 1(on next page)

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	12.92, 0.40	83.51, 0.16
10^6s[b]	13.15	83.33		
10^6c[c]	12.45	83.62		
10^5k	14.00	83.52	14.10, 0.17	83.48, 0.11
10^5s	14.00	83.35		
10^5c	14.30	83.57		
10^4k	15.45	83.56	16.30, 0.78	83.44, 0.11
10^4s	16.45	83.33		
10^4c	17.00	83.42		
10^3k	19.00	83.50	18.87, 1.35	83.45, 0.06
10^3s	17.45	83.39		
10^3c	20.15	83.47		
100k	20.15	83.47	20.35, 2.00	83.33, 0.21
100s	18.45	83.09		
100c	22.45	83.42		
10k	22.30	83.52	22.43, 1.50	83.42, 0.1
10s	21.00	83.42		
10c	24.00	83.33		
1k	23.15	83.52	23.92, 2.11	83.41, 0.12
1s	22.30	83.42		
1c	26.30	83.28		
0.1k	36.00	83.41	34.65, 1.91	83.39, 0.06
0.1s	-[d]	83.43		
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 to qPCR for organism detection using rapid swab processed samples with or without DNA extraction.

Table 7. Comparison of *C. pecorum* LAMP to qPCR for organism detection using rapid swab processed samples with or without DNA extraction.

Samples	Time to amplify (min)	Melt (°C)	qPCR copies/µl
K1 ocular swab RNA later	0.00	0.00	NEG
DNA	0.00	83.49	BDL (3)
K6 ocular swab RNA later	0.00	0.00	NEG
DNA	21.00	83.23	3x10^3
K9 ocular swab RNA later	0.00	0.00	NEG
DNA	25.45	83.39	287
K2 ocular swab RNA later	0.00	0.00	NEG
DNA	0.00	0.00	BDL (5)
R1 eye swab dry	25.45	83.39	222
R1 cloaca swab dry	30	83.34	NEG
K eye swab dry	27.00	83.15	NEG
Koala 2 eye swab dry	0.00	0.00	NEG
Koala 2 cloaca dry	27.30	83.77	116
Koala Will Cloaca dry	0.00	83.77	NEG
23117 Eye swab dry	21.30	83.20	NEG
23117 Cloaca swab dry	22.00	83.29	BDL (10)