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1 **Improving the molecular diagnosis of *Chlamydia psittaci* and *Chlamydia***
2 ***abortus* infection with a species-specific duplex real-time PCR.**

3

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16

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29 miscarriage

30 **Abstract**

31 *Chlamydia psittaci* and *Chlamydia abortus* are closely related intracellular bacteria exhibiting different
32 tissue tropism that may cause severe but distinct infection in humans. *C. psittaci* causes psittacosis, a
33 respiratory zoonotic infection transmitted by birds. *C. abortus* is an abortigenic agent in small ruminants,
34 which can also colonize the human placenta and lead to foetal death and miscarriage. Infections caused by
35 *C. psittaci* and *C. abortus* are underestimated mainly due to diagnosis difficulties resulting from their strict
36 intracellular growth. We developed a duplex real-time PCR to detect and distinguish these two bacteria in
37 clinical samples. The first PCR (PCR1) targets a sequence of the *16S-23S* rRNA operon allowing the
38 detection of both *C. psittaci* and *C. abortus*. The second PCR (PCR2) targets the coding DNA sequence
39 CPSIT_0607 unique to *C. psittaci*. The two PCR showed 100% detection for 10 or more DNA copies per
40 reaction (1000 copies per ml). Using a set of 120 samples including bacterial reference strains, clinical
41 specimens and infected cell culture material, we monitored 100% sensitivity and 100% specificity for the
42 detection of *C. psittaci* and *C. abortus* for PCR1. When PCR1 was positive, PCR2 could discriminate *C.*
43 *psittaci* from *C. abortus* with a positive predictive value of 100% and a negative predictive value of 88%.
44 In conclusion, this new duplex PCR represents a low cost and time saving method with high throughput
45 potential, expected to improve the routine diagnosis of psittacosis and pregnancy complication in large-
46 scale screening programs and also during outbreaks.

47

48 **Introduction**

49 *Chlamydia psittaci* and *Chlamydia abortus* belong to the family *Chlamydiaceae* that comprises obligate
50 intracellular bacteria. This family includes pathogenic members with specific host and tissue tropism
51 responsible for a broad range of diseases both in humans and animals (Corsaro & Greub 2006) (Baud *et al.*
52 2011) (Asner *et al.* 2014). *C. psittaci* is responsible worldwide for respiratory tract infections ranging from
53 benign illness to severe pneumoniae with fatal outcomes. The primary reservoir of *C. psittaci* is birds, in
54 particular parrots and parakeets (Longbottom & Coulter 2003) but recent studies on chicken from the food
55 industries and on feral pigeons suggest a significant prevalence of *C. psittaci* in these animals as well
56 (Magnino *et al.* 2009; Geigenfeind *et al.* 2012; Yin *et al.* 2013). Transmission between birds is primarily
57 achieved through the inhalation of aerosols containing desiccated infectious particles originating from
58 contaminated birds faeces. Infected birds can be asymptomatic hosts or develop respiratory tract disease,
59 namely ornithosis (Longbottom & Coulter 2003). The transmission from birds to humans can be achieved
60 through close interaction with infected birds or by inhalation of infected particles present in bird faeces
61 (Haag-Wackernagel & Moch 2004). The clinical expression of human psittacosis ranges from flu-like
62 illness to an atypical severe pneumonia (Longbottom & Coulter 2003). *C. abortus* can invade the placenta
63 and lead to abortions or foetal death in the late stage of pregnancy, especially in ruminants. In sheep, *C.*
64 *abortus*-induced disease is known as Ovine Enzootic Abortion (OEA), which is one of the principal causes
65 of lamb loss worldwide (Longbottom & Coulter 2003). *C. abortus* can also infect other mammals such as
66 goats, cattle, pigs, horses and deer with the same outcomes and can also be transmitted to humans through
67 contact with infected animal placentas that may contain a large quantity of infectious *C. abortus* organisms.
68 *C. abortus* infection is generally asymptomatic in non-pregnant animals but can colonize the placenta of
69 pregnant mammals, where it causes destruction of the placental tissue ultimately resulting in foetal death
70 and late-term abortion or miscarriage.

71 Both *C. psittaci* and *C. abortus* infections require rapid and accurate diagnosis in order to effectively
72 manage infected animals and humans and to limit or prevent their spread in animals given their epidemic

73 and zoonotic potential. Conventional diagnostic methods that include *in vitro* isolation of the organism,
74 immunohistochemistry and serology are time consuming have limited sensitivity and specificity (Sachse *et*
75 *al.* 2009).

76 We sought to improve, to accelerate and to simplify the diagnosis of *C. psittaci* and *C. abortus* infection by
77 using real-time PCR, which is a fast, sensitive and specific diagnostic method, in particular for intracellular
78 pathogens. Despite their distinct host and tissue tropism, *C. psittaci* and *C. abortus* are phylogenetically so
79 closely related that it is difficult to design a species-specific PCR targeting house-keeping genes, such as
80 the 16S rRNA gene (Pannekoek *et al.* 2010; Lienard *et al.* 2011). Thus, the aim of this study was to develop
81 a new real-time PCR-based method that could reliably detect and differentiate these two pathogens in
82 clinical samples that could be used in routine diagnostic laboratories.

83 We used a set of 120 samples, including bacterial reference strains, clinical specimens and DNA extracted
84 from infected cell cultures to determine the performance of our PCR. The sensitivity and specificity of
85 PCR1 for the detection of *C. psittaci* and *C. abortus* was 100%. When PCR1 is positive, PCR2 could
86 discriminate *C. psittaci* from *C. abortus* with a positive predictive value of 100% and a negative predictive
87 value of 88%. This new duplex PCR has been integrated into our automated molecular diagnostic platform
88 in a prospective manner and identified two cases of human *C. psittaci* infection. This duplex PCR is a rapid
89 and reliable method aimed to optimize the diagnosis of *C. psittaci* and *C. abortus* infections.

90 Material and methods

91 *Real-time PCRs design*

92 Taking advantage of the increasing number of available sequenced genomes of the family *Chlamydiaceae*
93 we designed a duplex real-time PCR to detect and to distinguish *C. psittaci* and *C. abortus* in clinical
94 samples. The first real-time PCR (PCR1) targets a region of the *16S-23S* rRNA operon. This PCR can detect
95 both *C. psittaci* and *C. abortus*. The second real-time PCR (PCR2) targets a recently identified coding DNA

96 sequence present only in *C. psittaci* (Voigt *et al.* 2012). All the primers and probes used for this study are
97 presented in table 1. Primers and probes were designed using the Geneious 7.1.7 and Primer3Plus software.
98 For PCR1, we performed an alignment of the 16S rRNA sequences available on GenBank and selected
99 primers specific to *C. psittaci* and *C. abortus* (Table 1). For the real-time PCR2, specific to *C. psittaci*, we
100 used the CDS CPSIT_0607 (GenBank Accession number NC_015470) predicted to encode for a protein of
101 yet undetermined function, and absent from the genomes of all other *Chlamydia*, including *C. abortus*.
102 Samples positive for both PCR1 and PCR2 were considered as positive for *C. psittaci*. When PCR1 was
103 positive and PCR2 was negative, genital samples or samples comprising abortion products were considered
104 positive for *C. abortus*. Considering that some strains of *C. psittaci* may not have the CDS CPSIT_0607,
105 respiratory samples positive for PCR1 and negative for PCR2 were considered positive for *C. psittaci*.

106 DNA from bacterial strains or clinical samples were extracted according to manufacturer's instructions with
107 the LC automated system (Roche, Rotkreuz, Switzerland) and the MagNA Pure LC DNA isolation kit I
108 (Roche) and resuspended into 100 µl of kit elution buffer.

109 The real-time PCRs, were performed with the ABI Prism 7900 Sequence Detection system (Applied
110 Biosystems, Rotkreuz, Switzerland). The reactions were achieved in a final volume of 20 µl with 5 µl of
111 DNA sample for 45 cycles, using primers and probes at the concentrations indicated in table 1 and the
112 TaqMan universal master Mix (Applied Biosystems). Cycling conditions were 2 min at 50°C, 10 min at
113 95°C, followed by 45 cycles of 15 s at 95°C and 1 min at 60°C. Plasmids containing the target amplicons
114 of the real-time PCR were obtained from RD-Biotech (Besançon, France). These plasmids were used as
115 positive controls and to determine the sensitivity limits and the reproducibility of the PCRs.

116 For each real-time PCR, a standard curve was generated by serial dilutions ranging from 5000 to 0.1 plasmid
117 copies. This allowed the quantification of positive samples in copies per PCR reaction, i.e. in copies per
118 five microliters of DNA.

119 ***Sensitivity, specificity and reproducibility of the duplex PCR***

120 The analytic performances were assessed individually for each PCR using the corresponding positive
121 control plasmids. The analytic sensitivity was performed with the positive control plasmids diluted from 50
122 to 0.01 DNA copies per reaction. We also determined that the duplexing of the PCR did not affect the
123 analytic sensitivity of the two PCRs. The intra- and inter-run reproducibility was assessed in duplicates in
124 5 independent runs with dilutions of the plasmids corresponding to 5000, 2000, 1000, 500, 200, 100, 50,
125 20, 10, 5, 2, 1, 0.5, 0.2 and 0.1 DNA copies per reactions.

126 For all subsequent steps of the development, the PCRs were always tested as a duplex PCR. The sensitivity
127 and specificity were tested using DNA from bacterial strains (Table 2), human (Table 4) and veterinary
128 (Table 5) clinical samples, as well as DNA from *Chlamydiales* species (Table 3).

129 ***Bacterial strains and samples***

130 To validate our new duplex PCR, we used a total of 120 samples which included 17 bacterial reference
131 strains (15 ATCC and 2 clinical isolates) (Table 2), 49 samples DNA samples obtained from cell culture
132 infected with *C. psittaci* (including all the 9 genotypes) and *C. abortus* and bacteria phylogenetically related
133 to these two organisms (Table 3), 22 human clinical samples matching the tissue tropism of *C. abortus* and
134 *C. psittaci* and 33 veterinary samples.

135 ***Gold standard and discrepant results investigation***

136 For the investigation of discrepant results, we designed the primers CPSI_OMP_F4 and CPSI_OMP_R1
137 for the amplification and the sequencing of the *ompA* gene of *C. psittaci* and *C. abortus* (Table 1). We also
138 used the *Chlamydiales*-specific real-time PCR developed by Lienard and colleagues that target the 16S
139 rRNA gene (Lienard, Croxatto et al. 2011). The PCR product of the *Chlamydiales*-specific real-time PCR
140 could be further sequenced using the primers panCh16F2 and panCh16R2. The *C. abortus* specific real-
141 time PCR targeting the *ompA* gene was used to detect *C. abortus* (Pantchev *et al.* 2009). The binding region
142 of the primer CPSI_00F, CPSI_00R and the probe CPSI_00P_MGB on the CDS CPSIT_0607 was

143 amplified using the primers CPSI607-SEQ-F1 and CPSI0607-SEQ-R3. The elongation was performed
144 using the primers CPSI_00F and the primer CPSI0607-SEQ-R3 (Table 1).

145 ***Case studies***

146 To further validate the new duplex real-time PCR, we analysed a number of local cases. Each positive
147 sample was analysed both with the *Chlamydiales*-specific real-time PCR as described by Lienard and
148 colleagues (Lienard, Croxatto et al. 2011) and with the new duplex real-time PCR.

149 **Results**

150 ***Target gene, primers and probes***

151 Despite different host specificity and tissue tropism, *C. psittaci* and *C. abortus* are phylogenetically closely
152 related bacteria. This prevents the differentiation of these two organisms by a single real-time PCR targeting
153 a housekeeping gene. Thus, we decided to develop a duplex PCR consisting of (i) a PCR (PCR 1) targeting
154 a house-keeping gene that allows the detection of both organisms but not their differentiation and (ii) a
155 second PCR (PCR 2) targeting a gene that allows their specific differentiation. The first PCR targets the
156 intergenic spacer of the 16S-23S rRNA operon (Table 1), while the second PCR targets the *C. psittaci* CDS
157 CPSIT_0607. A comparative genomic study has shown that CPSIT_0607 is unique to *C. psittaci* (Voigt,
158 Schofl et al. 2012).

159 ***Analytical sensitivity and reproducibility of the two real-time PCRs***

160 The sensitivity of the two real-time PCRs was evaluated with 5 replicates of different plasmid control
161 concentrations (50, 20, 10, 5, 2, 1, 0.5, 0.2, 0.1 copies per reaction). The PCR CP1 showed 100% detection
162 for 10 or more DNA copies per reaction, 90% for 5 and 2 copies per reactions and 60% for 1 copy per
163 reaction (Fig. 1A). The PCR 2 showed 100% detection for 5 or more DNA copies per reaction, 80% for 2
164 copies per reactions and 50% for 1 copy per reaction (Fig. 1B). The intra- and inter-run reproducibility of

165 PCR1 and PCR2 were assessed on 5 independent runs in duplicates using serial dilutions of the positive
166 control plasmid ranging from 5000 to 0.1 DNA copies per ml (Fig. 2). When plotting the cycle threshold
167 (Ct) of duplicates of the same amplification, we calculated a R^2 of 0.96 for PCR1 and 0.97 for PCR2, which
168 is excellent (Fig. 2A and 2B). To test the inter-run reproducibility we plotted the 10-copy and 100-copy
169 positive control of 10 successive runs, which revealed that only one 10-copy positive control exhibited a
170 Ct value that was above two standard deviation of the mean (Fig. 2C).

171 ***Performance of the duplex real-time PCR***

172 The overall performance of the duplex PCR was assessed with a total of 120 samples. No amplification was
173 observed with 17 bacterial strains (15 ATCC and 2 clinical isolates), including bacteria sharing the same
174 tissue tropism as *C. psittaci* or *C. abortus* (Table 2).

175 Forty-nine samples used to test the specificity of the PCRs were DNA samples (obtained from infected cell
176 culture material) from bacteria phylogenetically related to *C. psittaci* and *C. abortus*, namely *C. pecorum*
177 and *C. pneumoniae*, *Waddlia chondrophila*, *Estrella lausannensis*, *Criblamydia sequanensis*,
178 *Protochlamydia naegleriophila strain Knic* and *Simkania negevensis* (Table 3). The results of the duplex
179 PCR were correct for 42 of the 49 samples, with 8 results being discordant. Six of them correspond to DNA
180 samples obtained from cell cultures infected with *C. psittaci* genotype E/B, C and M56 that were found
181 positive for the PCR1 and negative for the PCR2 targeting the CDS CPSIT_0607. The analysis of the
182 genome of *C. psittaci* E/B revealed that this strain is devoid of the homolog of the CDS CPSI0607 (Van
183 Lent, Piet et al. 2012). The two other discordant results corresponded to samples thought to contain *C.*
184 *pecorum* DNA. These samples were positive for PCR1 and negative for PCR2 with our duplex PCR and
185 thus interpreted as positive for *C. abortus*. These two samples were further analysed both with the
186 *Chlamydiales*-specific PCR targeting the 16S rRNA sequence (Lienard, Croxatto et al. 2011) and a *C.*
187 *abortus* specific PCR targeting the *ompA* gene (Pantchev, Sting et al. 2009). Both PCRs were positive for
188 *C. abortus*, thereby confirming the presence of *C. abortus* DNA and validating our new duplex PCR.

189 The samples tested with the duplex PCR also included 22 human clinical samples, which selection was
190 biased towards genital and respiratory tract samples, matching the tissue tropism of *C. abortus* and *C.*
191 *psittaci* respectively. One sample was a uterus and urethral smear that was positive for *C. abortus*
192 corresponding to case 1 described above. The other samples were all negative for *C. psittaci* and *C. abortus*.
193 We obtained 100% concordance between our duplex PCR and the indicated gold-standard (Table 4). We
194 next tested the duplex PCR on 33 veterinary samples. The sample corresponding to a bird spleen positive
195 for *C. psittaci* (case 2 discussed above) was also identified as *C. psittaci* with our new duplex PCR. The 27
196 ovine placental samples positive for *C. abortus* (Longbottom *et al.* 2013), were all correctly identified by
197 our duplex PCR. No amplification was obtained with sample 1088D that was negative for both *C. abortus*
198 and *C. psittaci* according to our collaborator gold standard (Longbottom, Livingstone et al. 2013). In
199 contrast we obtained amplification with the PCR CP16 but not with the PCR 2 revealing the presence of *C.*
200 *abortus* DNA in samples 1086D, 1092D, 723P, 572P. These samples were found positive for *C. abortus*
201 with the Pan-*Chlamydiales* PCR and the *C. abortus* specific PCR targeting the *ompA* gene (Pantchev, Sting
202 et al. 2009) (Table 5).

203 On the basis of these 114 samples we determined 100% sensitivity and 100% specificity for our new *C.*
204 *psittaci* and *C. abortus* duplex PCR **(Table 6A)**. Moreover, when PCR1 was positive, PCR2 could
205 differentiate *C. psittaci* from *C. abortus* with a positive predictive value of 100% and a negative predictive
206 value of 91% **(Table 6B)**.

207 ***Case study***

208 The new duplex PCR was introduced into our diagnostic laboratory for a retrospective and prospective case
209 study analysis. Using our new duplex real-time PCR, we could confirm one case of ornithosis (Senn &
210 Greub 2008) and one case of *C. abortus* infection. We could also diagnose 2 cases of psittacosis. All these
211 cases are summarized in **table 7** and in **supplementary table 1**.

212 ***Optimisation of PCR1***

213 The CDS CPSI_0607 was chosen as the target for PCR2 on the basis of a comparison of the genome of *C.*
214 *psittaci* 6BC with the genomes of other *Chlamydiacae* species (Voigt, Schofl et al. 2012). Our *in silico*
215 analysis first identified 5 homologs of CPSIT_0607 in *C. psittaci* C19/98, *C. psittaci* 01DC11, *C. psittaci*
216 02DC15, *C. psittaci* 08DC60 (Schofl *et al.* 2011) and *C. psittaci* RD1 (Seth-Smith *et al.* 2011). The specific
217 primers and the probe for real-time PCR2 were designed using an alignment of these six sequences.
218 However, the sequencing of the target of the PCR 2 revealed a polymorphism (C147G) in the binding region
219 of the reverse primer in some *C. psittaci* positive samples. This mutation is predicted to induce a 5.8°C
220 decrease in the melting temperature (Tm) of the oligonuceotide CPSI_00R, but the cycle threshold (Ct) of
221 PCR1 and of PCR2 are similar in *C. psittaci* positive samples with the mutation C147G (see suppl. Table
222 S2). Thus, the mutation C147G does not impact the sensitivity of our duplex PCR. Nevertheless, we decided
223 to update our real-time PCR by using a combination of 2 reverse primers, namely the primer
224 CPSI_00R_147C and CPSI_00R_147G. We tested the duplex PCR containing this new primer on 5 *C.*
225 *psittaci* DNA samples containing the C147G mutation. The PCR containing the reverse primer
226 CPSI_00R_147C/G was as efficient at amplifying the target *C. psittaci* sequence as the PCR containing the
227 original primer CPSI_00R **(Suppl. table 2**).

228 **Discussion**

229 We have developed a new duplex real-time PCR which strength is to rapidly detect and distinguish *C.*
230 *psittaci* and *C. abortus* in clinical samples. The first PCR, which is based on the 16S rRNA gene, is able
231 to identify *C. psittaci* and *C. abortus* with 100% of sensitivity and specificity with a sensitivity limit lower
232 than 10 DNA copies per reaction. This PCR is suitable for the screening of *C. psittaci* and *C. abortus*
233 because of the 100% sensitivity for the two species. The second PCR that targets the CDS CPSI0607 found
234 in the genome of *C. psittaci* isolate 6BC and absent from all known *C. abortus* genomes allows
235 differentiating these two pathogens. The specific PCRs published to date for the detection of *C. psittaci* and
236 *C. abortus* target highly polymorphic genes, such as the *ompA* gene encoding the major outer membrane
237 protein (Pantchev, Sting et al. 2009). So far, none of the published *C. psittaci*/*C. abortus* real-time PCR

238 fulfilled the characteristic for an implementation in our molecular diagnosis platform based on the Taqman
239 technology and using standardized PCR in term of length of the amplicon and melting temperature of the
240 primers and probes and elongation temperature. This standardisation allows us to run all the real-time PCR
241 in batches in 384 multi-well plates. Branley et al have developed a PCR targeting the 16S rRNA gene but
242 using the SYBR-green technology and that presented a melting temperature lower than the target melting
243 temperature of our platform (60°C) {Branley, 2008 #631}. Most of the species specific real-time PCR target
244 outer membrane proteins with a high risk of loth of sensitivity due to single nucleotide polymorphism
245 {Creelan, 2000 #629}{Heddema, 2006 #301}{Heddema, 2015 #632}{Pantchev, 2009 #286}. The *incA*
246 gene encoding for the inclusion membrane protein A have been used to design a C. psittaci specific PCR
247 but published primers did not reach the Tm of our plateform {Menard, 2006 #25}. We recently developed
248 a Pan-*Chlamydiales* real-time PCR targeting the 16S rRNA able to detect with a high sensitivity (5 DNA
249 copies per reaction) and high specificity the presence of *Chlamydiales* species in clinical or environmental
250 samples. Nevertheless, when positive, a further sequencing of the PCR amplicons is required to identify the
251 bacteria at the species level (Lienard, Croxatto et al. 2011). Our new duplex PCR is a straightforward
252 method to rapidly detect and distinguish *C. psittaci* and *C. abortus* on molecular diagnosis platform using
253 the Taqman technology.

254 During the validation of our new duplex PCR, we found that the available genomes of *C. psittaci* genotype
255 A, B, D, E, F and WC contain orthologs of the CPSI0607 {Van Lent, 2012 #57}. In contrast the available
256 genomes of *C. psittaci* genotype E/B, identified in ducks, cattle and muskrats, genotype C, isolated from
257 ducks and geese and genotype M56, isolated in muskrats are devoid of this CDS (Geens *et al.* 2005; Van
258 Lent, Piet et al. 2012). The positive predictive value for the identification of *C. psittaci* is 100%, while the
259 negative predictive value is 88%. The analysis of more *C. psittaci* genomes will precise the distribution of
260 this gene. Thus, samples positive for both PCR1 and PCR2 can accurately be identified as containing *C.*
261 *psittaci* DNA. Conversely, genital samples or samples consisting of abortion products positive for PCR1
262 and negative for PCR2 were presumably identified as *C. abortus*. Respiratory samples positive for PCR1

263 and negative for PCR2 were considered as putatively positive for *C. psittaci*, especially in the context of
264 bird exposure.

265 During the validation of our new diagnosis methods, we also performed an *in silico* survey of the new
266 *Chlamydiacae* DNA sequences submitted together with the sequencing of the target gene of the PCR2 of
267 our duplex PCR. This allowed us to identify a polymorphism that could affect the binding of the reverse
268 primer of this PCR. This led us to design a degenerate primer to prevent any decrease in PCR sensitivity.
269 Our development is an example of the importance of a continuous *in silico* and *in vitro* survey of the
270 reliability of PCR-based molecular diagnosis methods to overcome the appearance of new pathogen
271 variants that could be missed. This is particularly true for pathogens for which the number of available
272 sequences is limited (*i.e.* emerging pathogens), or for pathogens with a high rate of mutations, such as
273 viruses.

274 The zoonotic incidence of *C. psittaci* and *C. abortus* is well documented but their incidence in human
275 diseases might be underestimated (Braukmann *et al.* 2012; Geigenfeind, Vanrompay et al. 2012). In
276 particular, about 50% of pneumonia cases remain diagnosed as of unknown etiology. *C. psittaci* should
277 especially be considered in the differential diagnosis if history reveals an exposure to birds. The situation
278 is similar for abortions, where nearly 50% of cases are of unexplained etiology (Baud *et al.* 2008) and could
279 sometimes be caused by *C. abortus*, particularly in rural areas or following exposure to sheep at lambing.
280 The sensitivity and the specificity of our new duplex PCR make it a powerful tool to confirm or to exclude
281 suspected *C. psittaci* or *C. abortus* infections during routine diagnosis. Moreover, the high throughput
282 potential together with the short turnaround time and the low cost of this real-time PCR make it a powerful
283 tool for large-scale screening programs during outbreaks, if such situations arise. This may be very
284 important, since the high infectivity of *C. psittaci* by the airway confers a significant outbreak potential
285 (Heddema *et al.* 2006; Gaede *et al.* 2008; Belchior *et al.* 2011; McGuigan *et al.* 2012) and consideration as
286 a potential bioterrorism agent.

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369

370

371 Table 1. Oligonucleotides sequences

Oligonucleotide	Target gene	Sequence (5'-3')	Modification/ fluorochrome	TM (°C)	Final concentration (µM)	Amplicon length (bp)	Specificity	References
Real time PCR								
CPSI_F	16S-23S operon	AAGGAGAGAGGCGCCCAA		59.7	0.35		*C. psittaci* and *C. abortus*	This study
CPSI_R_LNA	16S-23S operon	CAA{C}CTAGTCAAACCGTCCTAA	LNA {}	60	0.35	133	*C. psittaci* and *C. abortus*	This study
CPSI_P_MGB	16S-23S operon	ACTGGGATGAAGTCGTAAC	FAM, DQ	66.6	0.2		*C. psittaci* and *C. abortus*	This study
CPSI_00F	CDS CPSI0607	AGCATTAGCCAGCGCTTTAGA		58.1	0.35	118	*C. psittaci*	This study
CPSI_00R	CDS CPSI0607	TCTCTGAGCAAAAACACTGCGT		58.8	0.35	118	*C. psittaci*	This study
CPSI_00R_C147G	CDS CPSI0607	TCTCTCAGCAAAAACACTGCGT		53	0.35	118	*C. psittaci*	This study
CPSI_00P_MGB	CDS CPSI0607	ACAAAGACCTGGCGAGTA	VIC, DQ	67	0.2		*C. psittaci*	This study
panCh16F2 (a)	16S rRNA	CCGCCAACACTGGGACT		60	0.1		*Chlamydiales*	(Lienard, Croxatto et al. 2011)
panCh16R2 (a)	16S rRNA	GGAGTTAGCCGGTGCTTCTTTAC		60	0.1	207 to 215	*Chlamydiales*	(Lienard, Croxatto et al. 2011)
panCh16S	16S rRNA	CTACGGGAGGCTGCAGTCGAGAATC	FAM, BHQ	60	0.1		*Chlamydiales*	(Lienard, Croxatto et al. 2011)
CpaOMP1-F	*ompA*	GCAACTGACACTAAGTCGGCTACA		57.4	0.9		*C. abortus*	(Pantchev, Sting et al. 2009)
CpaOMP1-R	*ompA*	ACAAGCATGTTCAATCGATAAGAGA		52.8	0.9		*C. abortus*	(Pantchev, Sting et al. 2009)
CpaOMP1-Sb	*ompA*	TAAATACCACGAATGGCAAGTTGGTTTAGC G	FAM, TAMRA	60	0.2	82	*C. abortus*	(Pantchev, Sting et al. 2009)
PCR and sequencing								
CPSI_OMP_F4	*ompA*	GATCCTTGCGATCCTTGC		55.3			*C. psittaci* and *C. abortus*	This study
CPSI_OMP_R1	*ompA*	TGATAGCGGGACAAAAAGTTAGGA		60			*C. psittaci* and *C. abortus*	This study
CPSI607_SEQ_F1	CDS CPSI0607	ATGATTAACACAGCTATCGGC		56.6			*C. psittaci*	This study
CPSI607_SEQ_R3	CDS CPSI0607	ACTTGTTCCGCAGTTTGTTCCATC		62.3		393	*C. psittaci*	This study

372 LNA = Locked nucleic acid, MGB = Minor groove binder, FAM = 6-carboxy-fluorescein, VIC = TaqMan VIC reporter dye, DQ = Dark quencher,
373 BHQ = Black hole quencher, (a) also used for PCR and sequencing.

374

Table 2. Bacterial species used to test the specificity

Species	Source or strains
Escherichia coli	ATCC 25912
Klebsiella pneumoniae	ATCC BAA1706
Serratia marcescens	ATCC 8100
Pseudomonas aeruginosa	ATCC 27853
Haemophilus influenzae	ATCC 49247
Haemophilus paraphrophilus	ATCC 49917
Staphylococcus aureus	ATCC 29213
Staphylococcus epidermidis	ATCC 14990
Streptococcus pneumoniae	ATCC 49619
Streptococcus pyogenes	ATCC 19615
Streptococcus agalactiae	ATCC 12386
Streptococcus mitis	ATCC 6249
Enterococcus faecalis	ATCC 29212
Clostridium sporogenes	ATCC 19404
Lactobacillus spp. (1)	Clinical specimen
Bifidobacterium longum (1)	Clinical specimen
Candida albicans	ATCC 90028

(1) Identification using MALDI-TOF

375

376

377 Table 3. Bacterial DNA used to validate the duplex PCR

	Organism	*C. psittaci/C. abortus* duplex PCR result
Chlamydiales		
	Chlamydia psittaci 10298	*Chlamydia psittaci*
	Chlamydia psittaci 10525	*Chlamydia psittaci*
	Chlamydia psittaci 10/282	*Chlamydia psittaci*
	Chlamydia psittaci 10/423	*Chlamydia psittaci*
	Chlamydia psittaci 90/1051	*Chlamydia psittaci*
	Chlamydia psittaci 89/1291	*Chlamydia psittaci*
	Chlamydia psittaci 91/0154	*Chlamydia psittaci*
	Chlamydia psittaci 99_3005	*Chlamydia psittaci*
	Chlamydia psittaci 06/020	*Chlamydia psittaci*
	Chlamydia psittaci 06/052	*Chlamydia psittaci*
	Chlamydia psittaci 2000/332	*Chlamydia psittaci*
	Chlamydia psittaci 91/0237	*Chlamydia psittaci*
	Chlamydia psittaci 96_3218	*Chlamydia psittaci*
	Chlamydia psittaci 98_6098	*Chlamydia psittaci*
	Chlamydia psittaci 18_290800	*Chlamydia psittaci*
	Chlamydia psittaci 96_1867_30	*Chlamydia psittaci*
	Chlamydia psittaci 3_20901 (a)	*Chlamydia abortus*
	Chlamydia psittaci 4_20901 (a)	*Chlamydia abortus*
	Chlamydia psittaci 5_20901 (a)	*Chlamydia abortus*
	Chlamydia psittaci 2/290300 (a)	*Chlamydia abortus*
	Chlamydia abortus S263	*Chlamydia abortus*
	Chlamydia abortus 094_POS , Greek 'variant strain', ovine	*Chlamydia abortus*
	Chlamydia abortus 095_A22, ovine placenta	*Chlamydia abortus*
	Chlamydia abortus 096_S82/3, ovine placenta	*Chlamydia abortus*
	Chlamydia abortus 097_09/04, ovine placenta	*Chlamydia abortus*
	Chlamydia abortus 097_09/02, ovine placenta	*Chlamydia abortus*
	Chlamydia abortus 099_11/01, ovine placenta	*Chlamydia abortus*
	Chlamydia abortus 100_S135, ovine placenta	*Chlamydia abortus*
	Chlamydia abortus 101_S26/3, Moredun reference strain (sequenced), ovine placenta	*Chlamydia abortus*
	Chlamydia abortus 102_91/7, ovine placenta	*Chlamydia abortus*
	Chlamydia pecorum 093_P787, arthritogenic strain, ovine synovial fluid	*Chlamydia abortus (b)*
	Chlamydia pecorum 103_84/521F – ovine faecal strain	*Chlamydia abortus (b)*
	Chlamydia pecorum LW623	negative
	Chlamydia pecorum 66P130	negative
	Chlamydia pecorum 710S	negative
	Chlamydia pecorum L71	negative
	C.pneumoniae VR1310	negative
Parachlamydiales		
	Waddlia chondrophila	negative
	Parachlamydia acanthamoebae	negative
	Estrella lausannensis	negative
	Criblamydia sequanensis	negative
	Protochlamydia naegleriophila strain Knic	negative
	Simkania negevensis	negative

378 *(a) C. psittaci* genotype E/B (Van Lent, Piet et al. 2012)

379 *(b)* The presence of *C. abortus* DNA was confirmed by the pan-*Chlamydiales* PCR followed by sequencing (Lienard, Croxatto et
380 al. 2011) and by the real-time PCR *C. abortus* specific (Pantchev, Sting et al. 2009).

381 Table 4. Clinical specimen used to validate the duplex PCR

Sample type	Gold standard identification	*C. psittaci/C. abortus* duplex PCR result
Uterus and urethral smear	*Chlamydia abortus (a)*	*Chlamydia abortus*
Urethral swab	*Chlamydia trachomatis*	negative
Mouth swab (3 samples)	Oropharyngeal comensal flora	negative
Nasopharyngeal secretions (2 samples)	*Chlamydia pneumoniae*	negative
Nasopharyngeal secretions (2 samples)	*Mycoplasma pneumoniae (b)*	negative
Bronchoalveolar lavage (2 samples)	*Legionella pneumophila (b)*	negative
Nasal swab	*Bordetella pertussis*	negative
Nasopharyngeal secretions (2 samples)	*Bordetella pertussis*	negative
Expectoration	*Mycobacterium tuberculosis*	negative
Bronchoaspiration	*Mycobacterium tuberculosis*	negative
Bronchoaspiration	*Aspergillus fumigatus*	negative
Bronchoalveolar lavage	*Aspergillus fumigatus*	negative
Bronchoalveolar lavage	*Pneumocystis jirovecii*	negative
Bronchoaspiration	*Pneumocystis jirovecii*	negative
Drain fluid from aortic valve	*Coxiella burnetti*	negative
Prosthetic fragment	*Coxiella burnetti*	negative

382 *(a)* = pan-*Chlamydiales* PCR followed by sequencing (Lienard, Croxatto et al. 2011)

383 *(b)* = *M. pneumoniae, L. pneumophila* duplex real-time PCR (Welti *et al.* 2003)

384

385

386 Table 5. Veterinary specimens used for validation of the duplex PCR

Sample type	Gold standard identification	*C. psittaci/C. abortus* duplex PCR result
Bird spleen	*Chlamydia psittaci (a)*	*Chlamydia psittaci*
Fragment of sheep placenta	*Chlamydia abortus (a)*	*Chlamydia abortus*
Fragment of sheep placenta	*Chlamydia abortus (a)*	*Chlamydia abortus*
Biopsy 307A, placental cotyledonary	*Chlamydia abortus*	*Chlamydia abortus*
Biopsy 439P, placental cotyledonary	*Chlamydia abortus*	*Chlamydia abortus*
Biopsy 452A, placental cotyledonary	*Chlamydia abortus*	*Chlamydia abortus*
Biopsy 511P, placental cotyledonary	*Chlamydia abortus*	*Chlamydia abortus*
Biopsy 512P, placental cotyledonary	*Chlamydia abortus*	*Chlamydia abortus*
Biopsy 545A, placental cotyledonary	*Chlamydia abortus*	*Chlamydia abortus*
Biopsy 745P, placental cotyledonary	*Chlamydia abortus*	*Chlamydia abortus*
Biopsy 1725N, placental cotyledonary	*Chlamydia abortus*	*Chlamydia abortus*
Biopsy 2571N, placental cotyledonary	*Chlamydia abortus*	*Chlamydia abortus*
Biopsy 451P, placental cotyledonary	*Chlamydia abortus*	*Chlamydia abortus*
Biopsy 828P, placental cotyledonary	*Chlamydia abortus*	*Chlamydia abortus*
Biopsy 851P, placental cotyledonary	*Chlamydia abortus*	*Chlamydia abortus*
Biopsy 1504N, placental cotyledonary	*Chlamydia abortus*	*Chlamydia abortus*
Biopsy 1535N, placental cotyledonary	*Chlamydia abortus*	*Chlamydia abortus*
Biopsy 1683N, placental cotyledonary	*Chlamydia abortus*	*Chlamydia abortus*
Biopsy 2542N, placental cotyledonary	*Chlamydia abortus*	*Chlamydia abortus*
Biopsy 254B, placental cotyledonary	*Chlamydia abortus*	*Chlamydia abortus*
Biopsy 314A, placental cotyledonary	*Chlamydia abortus*	*Chlamydia abortus*
Biopsy 717A, placental cotyledonary	*Chlamydia abortus*	*Chlamydia abortus*
Biopsy 1019P, placental cotyledonary	*Chlamydia abortus*	*Chlamydia abortus*
Biopsy 1568N, placental cotyledonary	*Chlamydia abortus*	*Chlamydia abortus*
Biopsy 2537N, placental cotyledonary	*Chlamydia abortus*	*Chlamydia abortus*
Biopsy 637P, placental cotyledonary	*Chlamydia abortus*	*Chlamydia abortus*
Biopsy 758A, placental cotyledonary	*Chlamydia abortus*	*Chlamydia abortus*
Biopsy 1509N, placental cotyledonary	*Chlamydia abortus*	*Chlamydia abortus*
Biopsy 1088D, placental cotyledonary	*negative*	*negative*
Biopsy 1086D, placental cotyledonary	*negative*	*Chlamydia abortus**
Biopsy 1092D, placental cotyledonary	*negative*	*Chlamydia abortus**
Biopsy 723P, placental cotyledonary	*negative*	*Chlamydia abortus***
Biopsy 572P, placental cotyledonary	*negative*	*Chlamydia abortus***

387 *(a)* = pan-*Chlamydiales* PCR followed by sequencing (Lienard, Croxatto et al. 2011).

388 **C. abortus* DNA also detected with the Pan-Chlam, and with the *C. abortus* specific RT-PCR *ompA*.

389 ***infected animal, *C. abortus* DNA also detected with the Pan-Chlam, and with the *C. abortus* specific RT-PCR *ompA*.

390 Table 6. Performance of the duplex PCR. The duplex PCR was tested on 114 samples. The performance of
391 PCR1 that can detect *C. psittaci* and *C. abortus* was calculated on the 114. The performance of PCR2
392 specific of *C. psittaci* was calculated on the 61 positive for PCR1.

PCR1		*C. psittaci or C. abortus*	Other than *C. psittaci or C. abortus*		
	Correct for C. psittaci or C. abortus	65	0	100%	Positive predictive value
	Negative	0	49	100%	Negative predictive value
		100 %	100 %	114	
		Sensitivity	Specificity	total number of samples	

393

PCR2		*C. psittaci*	*C. abortus*		
	Positive	17	0	100%	Positive predictive value
	Negative	4	42	91%	Negative predictive value
		81%	100 %	61	
		Sensitivity	Specificity	total number of samples	

394

395

396 Table 7. Case study

	Sample	Interpretation of the duplex PCR *C. psittaci/C. abortus*	PCR1 result	PCR2 result	Clinical features
Case 1: *C. abortus* infection of a pregnant woman 2012 *(a)*	Uterus and urethral smear from a pregnant woman	*C. abortus*	Positive	Negative	Pregnant woman in contact with a flock of sheep
	Fragment of sheep placenta from an abortive sheep	*C. abortus*	Positive	Negative	Abortion
	Fragment of sheep placenta from a second abortive sheep	*C. abortus*	Positive	Negative	Abortion
Case 2: Parrot from a public park *(b)*	Parrot spleen	*C. psittaci*	Positive	Positive	Dead parrot from a public park dissected by an employee of a veterinary clinic that subsequently developed psittacosis
Case 3: Pigeon owner 2013 *(a)*	Expectoration from the pigeon owner	*C. psittaci*	Positive	Positive	Fever and hemoptysis. Radiologic condensations of the basoposterior segment of the right lower lobe of the lung in a patient in contact with a pigeon loft
	Pigeon choanal and cloacal swab	*C. psittaci*	Positive	Positive	Dead
	Dust from the pigeon loft	*C. psittaci*	Positive	Positive	-
Case 4: Worker on the roof of a building *(a)*	Endotracheal secretions	*C. psittaci*	Positive	Positive	Respiratory illness in a patient working on the roof of building

397 *(a)* This study

398 *(b)* (Senn & Greub 2008)

399

400 **Figure legends**

401 **Figure 1. Analytic sensitivity of the two real-time PCRs.** The analytic sensitivity was evaluated with 5
402 replicates of different plasmid control concentrations (50, 20, 10, 5, 2, 1, 0.5, 0.2, 0.1 copies per reaction)
403 for PCR1 (A) and PCR2 (B).

404 **Figure 2. Reproducibility of the two real-time PCR.** (A and B) Intra-run variability of the real-time PCR1
405 (A) and PCR2 (B) between duplicates of the control plasmid. (C and D) Inter-run variability using 10 and
406 100 plasmid copies, as obtained during 10 successive runs for PCR1 (A) and PCR2 (B); the solid black
407 lines show the mean cycle threshold (Ct) values and the dashed lines each side of the means indicates two
408 standard deviations.