

1 ***Clostridium perfringens*-associated necrotic enteritis-like disease in coconut**
2 ***lorikeets (*Trichoglossus haematodus*)***

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23 **Abstract**

24 Several outbreaks of necrotic enteritis-like disease in lorikeets, from which *C.*
25 *perfringens* was consistently isolated, are described. All lorikeets had acute,
26 segmental or multifocal fibrino-necrotizing inflammatory lesions in the small and/or
27 the large intestine, with intralesional gram-positive rods. The gene encoding *C.*
28 *perfringens* alpha toxin was detected by PCR on formalin-fixed, paraffin-embedded
29 tissues (FFPE) in 20 out of 24 affected lorikeets (83%), but it was not amplified from
30 samples of any of 10 control lorikeets ($p < 0.0001$). The second most prevalent *C.*
31 *perfringens* toxin gene detected was the beta toxin gene, which was found in FFPE
32 from 7 out of 24 affected lorikeets (29%). The other toxin genes were detected
33 inconsistently and in a relatively low number of samples. These cases seem to be
34 associated with *C. perfringens*, although the specific type involved could not be
35 determined.

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42 **Keywords:** Alpha toxin, beta toxin, *Clostridium perfringens* type A, lorikeet,
43 necrotizing enteritis, NetB toxin, *Trichoglossus haematodus*

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46 *Clostridium perfringens* is an important cause of enteric diseases in animals. The *C.*
47 *perfringens* species is currently divided into 7 types (A to G) on the basis of the
48 presence of 6 major toxin genes, i.e.: alpha (*cpa*), beta (*cpb*), epsilon (*etx*), iota (*itx*),
49 enterotoxin (*cpe*) and necrotic enteritis B-like toxin (*netb*).¹⁴ Type G strains, encoding
50 alpha- and NetB toxins, commonly cause necrotic enteritis (NE) in chickens and other
51 poultry species.⁷ Experimental and epidemiological evidence indicates that NetB
52 toxin is essential for development of NE.^{7,14} In addition, some authors suggest that *C.*
53 *perfringens* type A (that encodes alpha toxin [CPA]) and C (that encodes CPA and
54 beta [CPB] toxins) strains may also produce NE in poultry.¹² NE is a multifactorial
55 disease, and coccidiosis, stress, energy and protein-rich diets are known
56 predisposing factors in chickens.¹⁶ Our knowledge about *C. perfringens*-associated
57 enteritis in exotic birds is limited.^{2,3,5,6,10,11} It has previously been suggested that this
58 microorganism may cause an NE-like disease in both free-ranging and captive
59 lorikeets.^{6,10}

60 Between 2000 and 2018, 24 of the 67 (36%) lorikeets necropsied at the Institute of
61 Animal Pathology of the University of Bern were diagnosed with NE-like disease,
62 which represented the most frequent diagnosis. A consistent finding was the isolation
63 of *C. perfringens* from the intestine of affected birds. We here describe the pathologic
64 features of the disease and the results of PCR analyses for the detection of *C.*
65 *perfringens* major toxin genes on DNA extracted from formalin-fixed, paraffin-
66 embedded tissue of lorikeets.

67 The 24 coconut lorikeets (*Trichoglossus haematodus*) diagnosed with NE-like
68 disease belonged to a zoological institution (A, n=21) or a private aviary (B, n=3), and
69 had died spontaneously as part of five different outbreaks. Overall mortality in these
70 outbreaks ranged from 29 to 60%. None of the outbreaks were associated with

71 introduction of new birds, or other identifiable causes of stress. The clinical histories
72 included either sudden death or non-specific clinical signs such as apathy and
73 separation from the flock shortly before death. Diseased lorikeets included juveniles
74 (< 2 year-old, n=5), adults (\geq 2 year-old, n=13), and birds for which the age was not
75 recorded (n=6); the age range was 7 months to 9 years. There were equal numbers
76 of males and females. A full postmortem examination was performed and samples of
77 small and large intestine, lung, heart and kidney in all cases, and of brain, pancreas,
78 liver, spleen and skeletal muscle in most cases, were fixed by immersion in 10%
79 buffered formalin, pH 7.2, for 24 to 72 hours. Tissues were routinely processed for
80 histology and stained with hematoxylin and eosin (H&E); additional intestinal sections
81 were also stained with Gram. The necropsy reports were reviewed.

82 Gross lesions were observed in 14 of the 24 lorikeets (58%), and were limited to the
83 intestine. They consisted of segmental or multifocal, fairly well-demarcated
84 transmural hyperemia and/or hemorrhage, focally or multifocally ulcerated mucosa,
85 and multifocal to diffuse fibrinonecrotic membranes (Figure 1). Of the lorikeets that
86 had gross abnormalities, six had lesions exclusively in the small intestine (43%),
87 three (21%) had lesions only in the large intestine, and five (36%) had lesions in both
88 the small and the large intestine. This is in contrast with previous reports in lorikeets
89 and other exotic birds with NE-like disease, in which lesions were restricted to the
90 small intestine.^{6,10,15}

91 Despite gross lesions being reported in only 14 cases, all 24 lorikeets had
92 histologically moderate or severe fibrino-necrotizing lesions in the intestine (Figure 2).
93 The lesions were transmural in 13 (54%) cases, causing secondary peritonitis. The
94 superficial epithelium and the lamina propria had extensive areas of necrosis. A
95 fibrinonecrotic membrane composed of fibrin, viable and degenerate leukocytes,

96 blood and cellular debris covered the necrotic mucosa. Leukocyte infiltration of the
97 mucosa and submucosa, mostly heterophilic with fewer lymphocytes, plasma cells
98 and macrophages, was a prominent feature in 13 (54%) and mild in 11 (46%) birds.
99 The inflammatory cells formed a band between the viable and the non-viable tissue.
100 Myriad non-sporulated, gram-positive rods with morphology compatible with
101 *Clostridium spp.* were observed within the fibrinonecrotic membrane, lamina propria
102 and submucosa in all lorikeets (Figure 3). Fibrin thrombi were present in small
103 arterioles and venules of the mucosa and/or submucosa in 88% of the cases. In
104 addition, a few discrete foci of hepatocellular necrosis were randomly scattered
105 throughout the hepatic parenchyma in two lorikeets (8%). No other significant
106 microscopic lesions were observed in any bird. Overall, these lesions resembled
107 acute *C. perfringens*-induced NE in poultry.

108 Bacteriological investigations were initiated soon after the necropsies for 16 of the 24
109 lorikeets (Table 1). Intestinal contents were incubated anaerobically for 24 h at 37 °C
110 on membrane *C. perfringens* agar plates (mCP; Oxoid, Basel, Switzerland). Yellow,
111 circular, opaque colonies typical for *C. perfringens* were obtained in 12 cases (75%).
112 In seven of these cases, several of these colonies were pooled for DNA extraction
113 and PCR detection of *C. perfringens* toxin genes as previously described.¹ These
114 genes included *cpa*, *cpb*, *etx*, *iap*, *cpe* and *cpb2* (beta2 toxin). All samples were
115 positive for *cpa*. *cpb2* was detected in samples of two lorikeets (Table 1). PCR was
116 negative for the other toxin genes in all samples tested.

117 Causes of necrotizing enteritis in lorikeets include bacteria such as *Salmonella spp.*²⁰
118 and *C. colinum*,¹³ and parasites such as coccidia.¹⁷ Intestinal content from 16
119 animals was inoculated into enrichment in Muller-Kauffmann Tetrathionate-
120 Novobiocin Broth (Oxoid, Ref: BO1224K) followed by subculture on Brilliance

121 *Salmonella* (Oxoid, Ref: PO5098A) and Brilliant Green Agar (Modified) (Oxoid, Ref:
122 PO5033A). No *Salmonella* spp. were isolated in any of these 16 cases. No parasites
123 were detected by using a combined sedimentation-flotation method with ZnCl₂ on
124 intestinal contents in three lorikeets. No coccidia were detected on histological
125 sections of any of the birds. Because no specific medium for *C. colinum* was used, a
126 co-infection by this micro-organism cannot be ruled out.¹³

127 Because the above-mentioned *C. perfringens* toxinotyping PCR protocol was only
128 performed on isolates from a subset of the lorikeets and did not include *netb*, we
129 retrospectively evaluated the presence of this toxin gene and the other typing toxin
130 genes¹⁴ on DNA extracted from FFPE intestinal samples of all lorikeets affected by
131 NE-like disease and from 10 control lorikeets without necrotizing intestinal lesions.
132 Total DNA was extracted from three 10-µm thick paraffin sections using a QIAmp
133 DNA FFPE Tissue Kit (Qiagen, Hilden, Germany). Primers that were specific for short
134 fragments of the main *C. perfringens* toxin genes were designed (Supplemental
135 Table S1). DNA extracted from FFPE intestinal sections, from which the
136 corresponding *C. perfringens* type had been isolated, was used as positive control.
137 PCR amplicons were visualized in ethidium bromide-stained 1% agarose gels
138 (Agarose SFP; Amresco, Solon, Ohio). Because all strains of *C. perfringens* produce
139 CPA, the amplification of *cpa* was considered indicative of the presence of *C.*
140 *perfringens* DNA in the sample. *Cpa* was detected in 20 of the 24 lorikeets affected
141 by necrotizing intestinal lesions (83%) (Table 1), but in none of the 10 control
142 lorikeets (p=0.0001, Chi-square with Yates' correction, GraphPad Prism, San Diego,
143 California, USA). This is in agreement with previous data indicating that *C.*
144 *perfringens* is uncommonly found in healthy lorikeets or other psittacines.¹⁵ *C.*
145 *perfringens* was, however, isolated from the intestine of two lorikeets negative for *cpa*

146 in the FFPE samples. Thus altogether, *C. perfringens* was detected in the intestine of
147 22/24 lorikeets (92%) affected by necrotizing intestinal lesions. In 10 (42%) of the
148 lorikeets with NE-like disease, *cpa* was the only toxin gene detected. The second
149 most prevalent *C. perfringens* toxin gene detected was *cpb* (29%), while the other
150 toxin genes were detected inconsistently and in a relatively low number of samples
151 (Table 1).

152 The role of CPA in intestinal diseases of animals has been suggested but never
153 definitively proven,^{4,19} and there is no evidence to conclude from our results that CPA
154 was responsible for the lesions observed in our birds. We cannot however,
155 completely rule out a role for this toxin in the pathogenesis of the NE-like disease.
156 CPB is responsible for necrotizing enteritis in several animal species including
157 birds,^{12,16} and the lesions described in these lorikeets were very similar to those
158 described in mammals and birds affected by this toxin.^{12,18} It is therefore possible
159 that CPB was responsible for the necrotizing intestinal lesions observed in at least
160 some of these lorikeets. This is in agreement with a previous report that identified
161 CPB in the intestine of lorikeets with necrotizing enteritis.¹⁰ While NetB-producing
162 type G strains have been shown to play a major role in NE in chickens and other
163 poultry species,⁷ *netB* was detected in only one lorikeet in this study.

164 In summary, our results suggest that, in lorikeets, a disease similar to the NE from
165 chickens is associated with the presence of *C. perfringens* in the intestine of the
166 lorikeets. *C. perfringens* type A was identified in 83% of lorikeets with NE-like
167 disease. We could not, however, conclusively demonstrate a role of a *C. perfringens*
168 toxin in the pathogenesis of this disease. It is possible that other yet unknown toxins
169 contributed to the necrotizing intestinal lesions in these lorikeets.¹⁸ Similarly, CPA
170 was considered the key virulence factor for NE in broiler chickens for many years

171 until recent evidence demonstrated that NetB, and not CPA, is the main virulence
172 factor of NE-producing type G strains.⁷ NetF-positive type A strains may be involved
173 in canine hemorrhagic gastroenteritis and equine necrotizing enteritis, although
174 definitive evidence of the role of NetF in these diseases is lacking.⁹ Moreover,
175 several previously unknown toxin genes were identified in isolates from turkeys,⁸
176 indicating a much more diverse picture of pathogenic *C. perfringens* type A isolates.
177 However, most studies (including ours) lack consistent isolation and full
178 characterization of *C. perfringens* isolates from the intestine of diseased animals.
179 Fulfillment of Koch's postulates is also lacking. Therefore, conclusions on a causal
180 relationship of particular pathogenic strains of *C. perfringens* type A with NE-like
181 disease in lorikeets or other exotic birds cannot be drawn. To investigate the causal
182 relationships of different *C. perfringens* with NE-like disease in animals, whole-
183 genome sequencing of *C. perfringens* isolates should be considered, along with
184 experimental work to fulfill Koch's postulates.

185 **Acknowledgements**

186 We kindly thank Ms S.J. Uzal for critical review of this manuscript.

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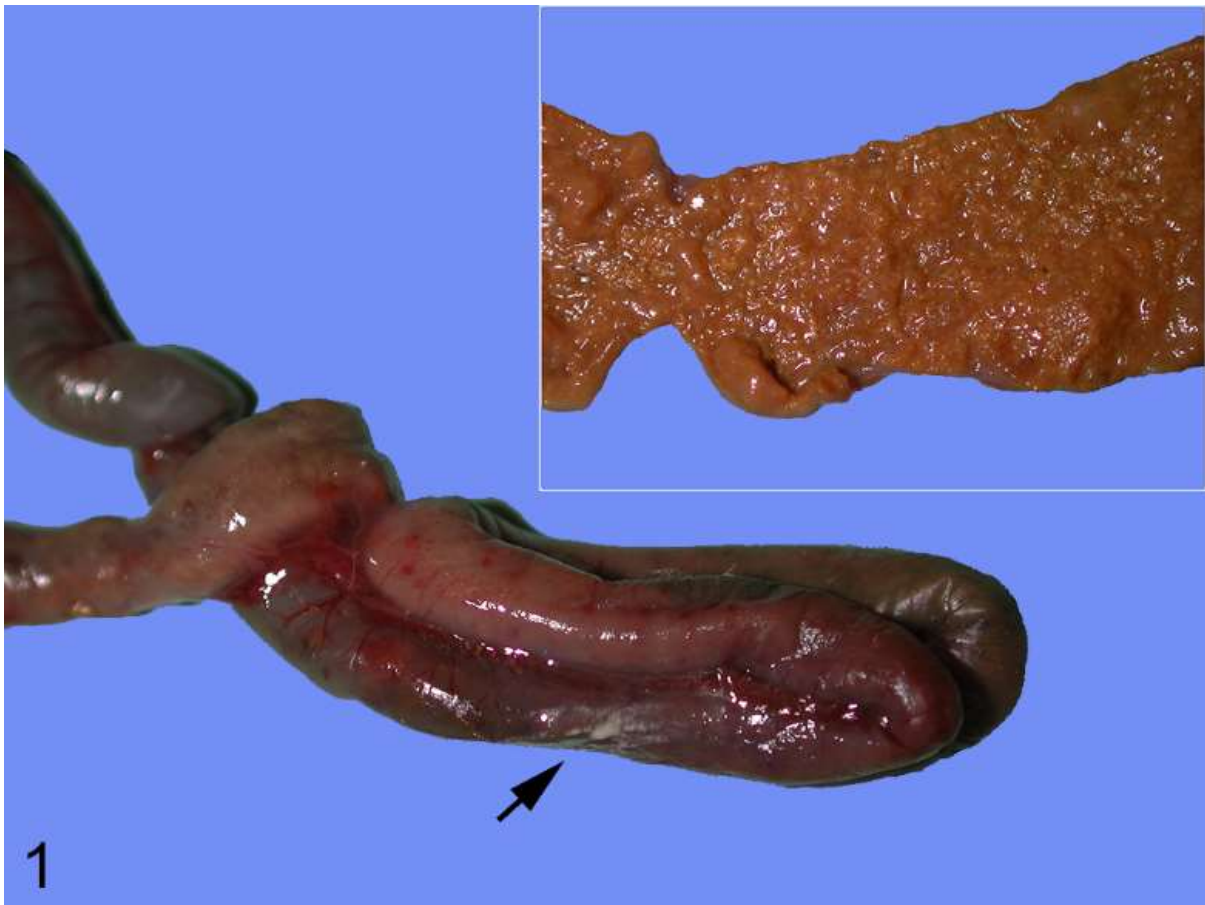
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265 **Figure Legends**

266 **Figure 1.** Necrotizing enteritis, small intestine, lorikeet. There is multifocal reddening
267 of the intestinal wall, which corresponds to necrotizing enteritis visible from the
268 serosal surface. The intestinal serosa has multifocal white areas, which correspond
269 to areas of transmural inflammation and necrosis (peritonitis, arrow). Inset: The
270 mucosa is diffusely necrotic.

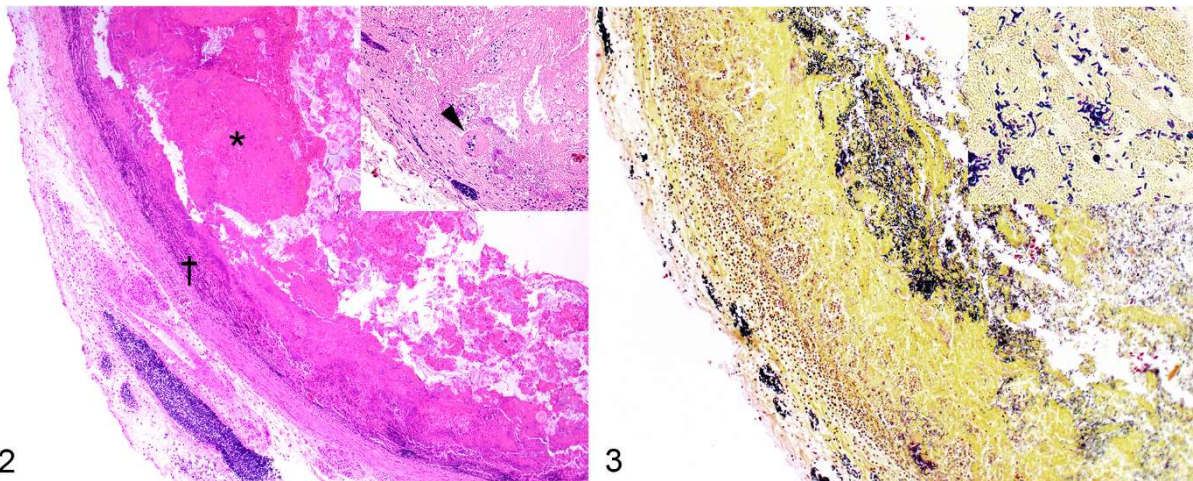


277 **Figure 2.** Transmural fibrinonecrotizing enteritis, small intestine, lorikeet. The
278 mucosa is diffusely necrotic and covered by a fibrinonecrotic membrane (asterisk).
279 There is loss of the mucosal/submucosal boundary and transmural infiltration with
280 inflammatory cells (dagger). Inset: The mucosa contains a fibrin thrombus within a
281 small vessel (arrowhead).

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283 **Figure 3.** Small intestine, lorikeet. Abundant gram-positive rods are present within
284 the necrotic mucosa and the intestinal lumen. Inset: Bacillary morphology of the
285 gram-positive Clostridial-like bacteria in the necrotic mucosa.

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294 **Table 1.** Results of *Clostridium perfringens* culture and PCR typing in 24 lorikeets with necrotic enteritis-like disease.

Outbreak Number and Origin (A or B)	Animal Number	<i>Clostridium perfringens</i> isolation	PCR on FFPE intestine and on isolates (in brackets) ^a							Inferred Possible Toxinotypes ^b	
			<i>cpa</i> (alpha toxin)	<i>cpb</i> (beta toxin)	<i>etx</i> (epsilon toxin)	<i>iap</i> (iota toxin)	<i>cpe</i> (CPE)	<i>netB</i> (NetB)	<i>cpb2</i> (beta2 toxin)		
1 (A)	1	-	+	-	-	-	-	-	-	NP	A
1 (A)	2	NP	+	+	-	-	-	-	-	NP	A, C
1 (A)	3	+	+	-	-	-	-	+	+	NP	A, F, G
1 (A)	4	-	+	-	-	-	-	-	-	NP	A
1 (A)	5	NP	-	-	-	-	-	-	-	NP	-
1 (A)	6	-	-	-	-	-	-	-	-	NP	-
1(A)	7	+	-	-	-	-	-	-	-	NP	-
1 (A)	8	+	-	-	-	-	-	-	-	NP	-
2 (B)	9	+	+	-	-	-	-	-	-	NP	A
2 (B)	10	-	+	-	-	-	-	-	-	NP	A
3 (B)	11	NP	+	+	+	-	-	-	-	NP	A, C, D
4 (A)	12	+	+(+)	+(-)	-(-)	-(-)	-(-)	-(-)	-	NP (-)	A, C
4 (A)	13	NP	+	-	-	-	-	-	-	NP	A
4 (A)	14	NP	+	-	-	-	-	-	-	NP	A
4 (A)	15	NP	+	-	-	-	-	-	-	NP	A
4 (A)	16	+	+(+)	-(-)	-(-)	-(-)	-(-)	-(-)	-	NP (-)	A
4 (A)	17	NP	+	+	+	-	+	-	-	NP	A, B, C, D, F
4 (A)	18	+	+	+	+	-	+	-	-	NP	A, B, C, D, F
4 (A)	19	NP	+	+	+	-	-	-	-	NP	A, C, D
4 (A)	20	+	+(+)	-(-)	-(-)	-(-)	-(-)	+(-)	-	NP (-)	A, F
5 (A)	21	+	+(+)	-(-)	-(-)	-(-)	-(-)	-(-)	-	NP (-)	A
5 (A)	22	+	+(+)	-(-)	-(-)	-(-)	-(-)	-(-)	-	NP (-)	A
5 (A)	23	+	+(+)	-	-	+	-	-	-	NP (+)	A, E
5 (A)	24	+	+(+)	+	+	+	+	-	-	NP (+)	A, B, C, D, E, F
TOTAL [Percentage]		12/16 [75%]	20/24 [83%]	7/24 [29%]	5/24 [21%]	2/24 [8%]	4/24 [17%]	1/24 [4%]		2/7 [29%]	

295 Abbreviations: CPE, *Clostridium perfringens* Enterotoxin; FFPE, formalin-fixed, paraffin-embedded; NetB, necrotic enteritis B-like; NP,
296 not performed.

297 ^aPCR testing was done on FFPE intestine from all lorikeets, and from the bacterial isolates in 7 of the animals. PCR results are given
298 as + (positive) and – (negative). PCR results on bacterial isolates is indicated in brackets. Two discrepant PCR results are highlighted
299 in bold.

300 ^bThe *C. perfringens* toxinotypes possibly involved in each lorikeet are listed.

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311 **Supplemental Table 2.** Primers used for detection of the genes encoding the typing
 312 toxins of *C. perfringens* in formalin-fixed, paraffin-embedded sections of intestinal
 313 tissue.

Primer name	Sequence (5'-3')	Target gene	Product size (bp)
CPAF	AAGGCGCTTATTTGTGCCG	<i>cpa</i>	101
CPAR	GCATGAGTTCCTGTTCCATCA	(alpha toxin)	
CPBF	GCGAATATGCTGAATCATCTA	<i>cpb</i>	196
CPBR	GCAGGAACATTAGTATATCTTC	(beta toxin)	
ETXF	GAAGTGAATGGGGAGAGATACCTA	<i>etx</i>	160
ETXR	ATTAACTCATCTCCCATAACTGCAC	(epsilon toxin)	
ITXF	TTGTATATAGAAGGTCTGGTCCAC	<i>iap</i>	127
ITXR	GGGTATGTTACTTTTCCTTCCC	(iota toxin)	
CPEF	TGGATATTAGGGGAACCCTCAG	<i>cpe</i>	227
CPER	TTTGGACCAGCAGTTGTAGATA	(enterotoxin)	
NetBF	ATCCTCATTCTGATAAGAAAAGTGC	<i>netB</i>	250
NetBR	TTTCCTTCAACAGATATATTACCGC		

314 PCR performed in a total volume of 25 μ L containing 0.5 μ L of each primer (0.5 μ M),
 315 5 μ L of extracted DNA, 7 μ L of nuclease-free water and 12 μ L of PCR Master Mix 2X
 316 Promega (Madison, Wisconsin). Thermocycler profiles were as follows: 95°C for 10
 317 minutes, 35 cycles of 95°C for 35 seconds, 50°C for 35 seconds, and 72°C for 35
 318 seconds, and a final extension step at 72°C for seven minutes.

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