Vol. 129: 199–205, 2018
https://doi.org/10.3354/dao03244

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published August 14

PCR test to specifically detect the apicomplexan 'X' (APX) parasite found in flat oysters *Ostrea chilensis* in New Zealand

Nguyen Thao Suong[1,2,*], Jonathan C. Banks[2], Stephen C. Webb[2], Andrew Jeffs[1], Kevin C. Wakeman[3,4], Andrew Fidler[1]

[1]Institute of Marine Science, University of Auckland, Auckland 1142, New Zealand
[2]Cawthron Institute, Nelson 7010, New Zealand
[3]Office of International Affairs, Hokkaido University, Sapporo 060-0810, Japan
[4]Faculty of Science, Hokkaido University, Sapporo 060-0810, Japan

ABSTRACT: Described here is a polymerase chain reaction (PCR) test to detect the apicomplexan-X (APX) parasite of a flat oyster species, *Ostrea chilensis*, endemic to New Zealand. The test primers target sequences in the *in situ* hybridisation probes identified to bind specifically to APX 18S rRNA and amplify a 723 bp DNA product. The test did not amplify 18S rRNA gene sequences of other apicomplexan species, including *Toxoplasma gondii*, *Neospora caninum*, *Selenidium* spp., *Cephaloidophorida* spp., *Lecudina* spp. and *Thiriotia* sp. Of 73 flat oysters identified by histology to be infected with APX at different severities, 69 (95%) tested PCR-positive. Failure to amplify an internal control indicated the presence of PCR inhibitors in the 4 PCR-negative samples. The high analytical sensitivity, specificity and speed of the PCR test should make it a useful tool for detecting APX.

KEY WORDS: Apicomplexan · Parasite · *Ostrea chilensis* · New Zealand

INTRODUCTION

Shellfish aquaculture contributed 31% of all seafood consumed in 2015 and is expected to grow to meet the future demand (FAO 2017). However, microbial diseases caused by protozoans (e.g. *Marteilia* spp., *Perkinsus* spp. and *Bonamia* spp.), bacteria (e.g. *Vibrio parahaemolyticus*, *Flexibacter* sp.) and viruses (e.g. white spot syndrome virus [WSSV], ostreid herpes virus) continue to hinder industry growth (Doonan et al. 1994, Chou et al. 1995, Tajima et al. 1997, Raftos et al. 2014, Lee et al. 2015). These pathogens may infect a single species, a group of related species (e.g. *Marteilia sydneyi* in Sydney rock oysters), a broad range of species (e.g. *Perkinsus olseni*, WSSV) (Raftos et al. 2014), all shellfish life stages (e.g. *Vibrio harveyi*) (Austin & Zhang 2006, Mine & Boopathy 2011) or a specific life stage (e.g. *Vibrio tubiashii*) (Elston et al. 2008) and can cause devastating disease epizootics. As examples, pathogenic diseases result in up to 40% (>$3 billion USD) of annual production losses to global shrimp aquaculture (Stentiford et al. 2012) and have caused epizootics resulting in >90% stock depletion among oyster species such as *Crassostrea virginica* reared in Chesapeake Bay, USA (Guo & Ford 2016), *Ostrea edulis* reared in Brittany, France (Naciri-Graven et al. 1998) and *Saccostrea glomerata* reared on the east coast of Australia (Adlard & Nolan 2015).

The flat oyster *Ostrea chilensis* provides the basis of the oldest commercial fishery in New Zealand and is worth ~$20 million USD annually (Velvin 2015).

*Corresponding author: suongthaonguyenvn@gmail.com

Parasitic diseases, particularly bonamiosis caused by *Bonamia* spp., represent the greatest threat to *O. chilensis* and resulted in the collapse of an emerging industry to farm this species (Doonan et al. 1994, Diggles et al. 2002, Lane et al. 2016). *Bonamia* infection in flat oysters is often accompanied by infection with an uncharacterised apicomplexan-'X' or APX parasite (Hine 2002a, Lane et al. 2016). Oyster co-infection by *Bonamia exitiosa* and APX has been associated with dramatically reduced population numbers in commercial fisheries, such as occurred in Foveaux Strait between 1985 and 1993 (Diggles et al. 2002). This concurrent infection is thought to be synergistically detrimental to the host, as both *B. exitiosa* and APX are associated with depletion of the host glycogen reserve (Hine 2002a). Severe infections of APX otherwise occur in apparently healthy flat oysters and may predispose those oysters to bonamiosis (Diggles et al. 2002). Despite the pathogenic threat posed by APX, its life cycle, infection mechanisms and seasonal prevalence remain largely unknown.

Histopathology has revealed a high prevalence of APX zoites in flat oysters (Diggles et al. 2002, Hine 2002a). For example, examination of 6455 oysters sampled from Foveaux Strait between 1986 and 1991 identified >85% to be infected with APX (Hine 1991). In addition, high APX infection loads appeared to affect oyster gametogenesis, with 9% of high-load oysters having empty gonad follicles lacking germinal epithelium compared with only 2% of moderate-load and 1% of low-load oysters (Hine 2002a). Furthermore, vesicular connective tissue in oysters heavily parasitised by APX was found to be severely damaged, likely leading to host sterility and death (Hine 2002a).

In addition to APX in flat oysters, zoite life-stage apicomplexans with similar unique morphology (slipper-shaped with a central nucleus and posterior amylopectin granules) have been detected in the green-lipped mussel *Perna canaliculus* from the Marlborough Sounds in New Zealand (Diggles et al. 2002, Hine 2002b, Webb 2008) and more recently in the Pacific oyster *Crassostrea gigas* and blue mussel *Mytilus edulis* in New Zealand (S. C. Webb unpubl. data). These commercially important bivalve species may be hosts for APX, however, histology evidence relies primarily on apicomplexan morphology which may be non-specific, for example if those apicomplexans supposed to be APX are cryptic species. Furthermore, histology is costly and time-consuming.

To detect APX more specifically and efficiently, a 1.8 kbp region of the APX 18S rRNA gene was recently amplified from APX-infected *O. chilensis* and used to generate APX 18S rRNA-specific *in situ* hybridisation probes (Suong et al. 2017). Building on this, we describe a sensitive and specific PCR test for the APX 18S rRNA gene derived from APX-specific primer sequences designed for the *in situ* hybridisation probes. PCR analytical specificity was confirmed by testing 16 specimens of other apicomplexan DNA extracted from a range of hosts, analytical sensitivity (detection limit) was determined using DNA dilution series and diagnostic sensitivity was determined by comparing PCR data and histological data for 75 flat oysters infected with APX at varying severities estimated by histology.

MATERIALS AND METHODS

Samples

Oysters (n = 75) were collected from Foveaux Strait, New Zealand. After measuring oyster length and cleaning off excess external shell debris, each oyster was opened using a sterilised knife and a transverse section (3 to 5 mm thick) containing gill, mantle and visceral mass was cut and stored frozen at −70°C in a 5 ml tube. For histology, another comparable tissue section was placed into a labelled histology cassette and fixed for 48 h in 4% formaldehyde in seawater before transfer to 70% ethanol.

Histology

Fixed oyster specimens were processed and tissue sections were stained with hematoxylin and eosin at Taranaki Medlab (New Plymouth, NZ). As described by Gagné et al. (2015) with some modifications, each tissue section was viewed at low magnification (100 to 200×) to confirm specimen quality, at intermediate magnification (400×) to detect lesions, host responses and the presence of other pathology and at high magnification (1000×) to detect APX. Under high magnification, 25 fields of both visceral mass (digestive gland, gut and connective tissue) and gill tissue were examined. Each field-of-view was graded from 0 to 3 (Hine 2002a), where Grade 0 = no APX observed; Grade 1 = <5 parasites (light infection); Grade 2 = parasites easily observed, particularly around the haemolymph sinuses (light to moderate infection); and Grade 3 = parasites abundant in all tissues especially among and within connective tissues, and with many lesions evident (moderate to

heavy infection). Tissue sections were viewed 3 times to ensure grading consistency.

PCR primer sequences

The APX 18S rRNA PCR test primers APX-For (5′-TCT TTG AGT GAG AAT CCG GTT TG-3′) and APX-Rev (5′-GTG AGT CGA GAA CAA AGA ACA TTC-3′) were designed to target unique sequences of specific ISH-APX-Pr01 and ISH-APX-Pr02 *in situ* hybridisation probes identified in alignments of APX 18S rRNA gene to 18S rRNA gene sequences of the flat oyster, Pacific oyster *Crassostrea gigas*, *Bonamia ostreae* and *Bonamia exitiosa* (Fig. 1). Each primer sequence was also subjected to an independent Basic Local Alignment Search Tool (BLAST) search to help ensure specific amplification of APX DNA.

DNA extraction and PCR

DNA was extracted from 20 to 25 mg of flat oyster gill and digestive gland tissue using a commercial kit (Zymo Genomic DNA Tissue Mini Prep Kit) following the manufacturer's instructions. Each PCR (20 µl final volume) was comprised of 10 µl 2 × MyFi Mix (Bio-Line), 10 pmol of each APX-For and APX-Rev primer and either 1 µl template DNA (150 ng) or ultra-pure distilled water (Invitrogen) as a no-template control. Thermal cycling conditions were 95°C for 2 min, 35 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 30 s, 72°C for 5 min and hold at 15°C. Amplified DNA (723 bp) was resolved in a 1% (w/v) agarose gel containing 0.5 µg ml^{-1} ethidium bromide and visualised using a UV transilluminator. Any PCR product of the expected size (723 bp) was purified using the Nucleospin® gel and PCR clean-up kit (Macherey-Nagel),

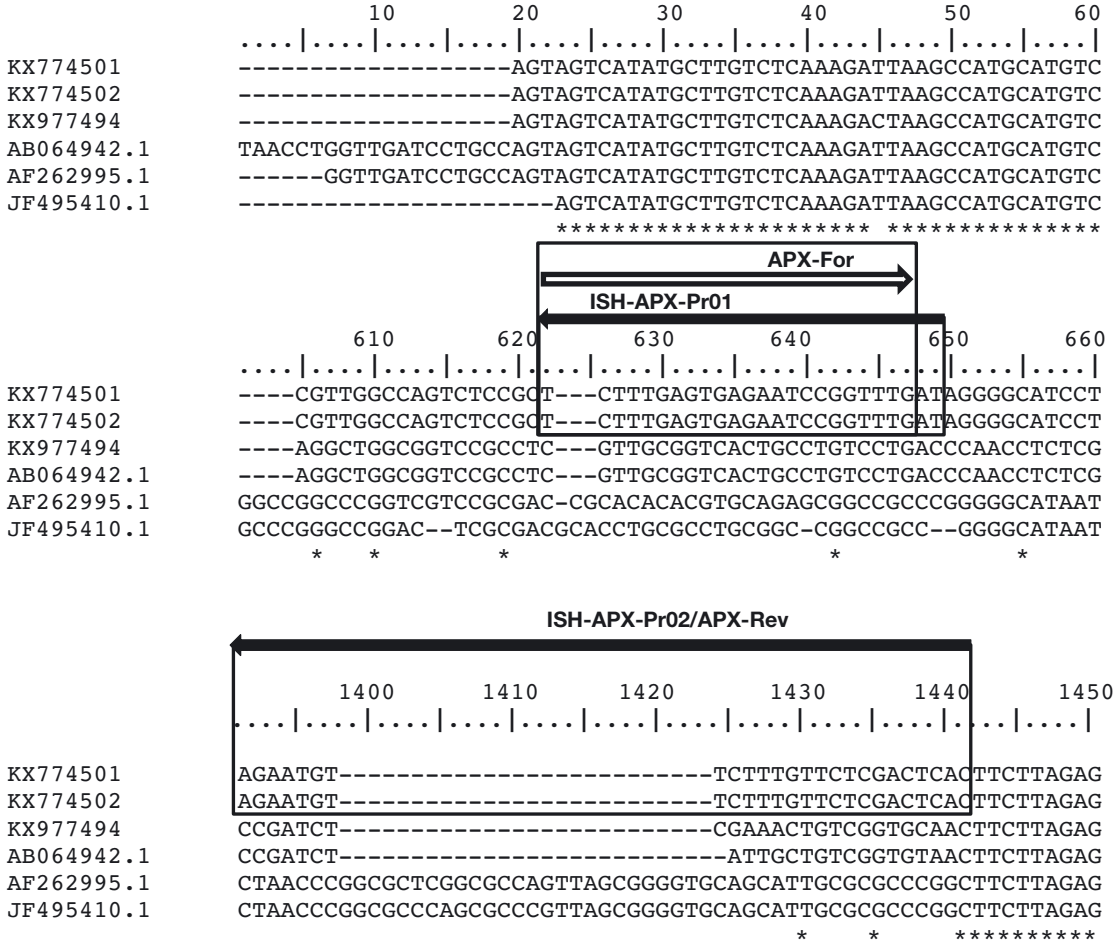

Fig. 1. Location of PCR primers APX-For and APX-Rev based on *in situ* hybridisation probes ISH-APX-Pr01 and ISH-APX-Pr02 that specifically bind to apicomplexan-X (APX) cells within infected flat oyster tissues. Two *in situ* hybridisation probes (ISH-APX-Pr01 and ISH-APX-Pr02) were designed based on 2 regions discriminating the APX 18S rRNA sequences (KX774501, KX774502) from the flat oyster (KX977494), Pacific oyster (AB064942), *Bonamia ostreae* (AF262995) and *Bonamia exitiosa* (JF495410) partial 18S rRNA sequences. Note bases 61–599 and 661–1390 are omitted for clarity

ligated into pGEM-T Easy vector (Promega) and pDNA clones containing inserts were sequenced by an external contractor (Massey Genome Service). All sequences were used to interrogate GenBank for similar sequences using BLAST.

To determine the analytical sensitivity of the PCR test, the primer set 3011For1 and SSUR4 (Suong et al. 2017) was used to produce a 1296 bp APX 18S rRNA gene DNA product. The 1296 bp APX 18S rRNA gene DNA product amplified by PCR was purified as above, quantified by UV spectrophotometry and diluted to a concentration of 100 ng μl^{-1} in sterile distilled water. This DNA was used to prepare serial 10-fold dilutions down to 1 ag μl^{-1} that were then mixed 1:1 with DNA (150 ng μl^{-1}) from a flat oyster confirmed to be APX-free by both PCR and histology. DNA dilutions were then amplified in triplicate with the APX 18S rRNA PCR test primers APX-For and APX-Rev, and a 10 µl aliquot of each 20 µl PCR was analysed by gel electrophoresis. The lowest dilution 0.1 fg μl^{-1} generating a visible 723 bp DNA band in all 3 replicate reactions was converted to dsDNA copy number based on the sequence length or mass using DNA/RNA copy number calculator tool (www.endmemo.com/bio/dnacopynum.php; accessed 28 Jan 2018). As 2 or more criteria generally need to be met to diagnose a pathogen with confidence (Ramis et al. 2012), the diagnostic sensitivity and specificity of the PCR test was estimated based on histology as the reference method.

To examine PCR test analytical specificity, DNA extracted from 16 apicomplexan species spanning 6 genera were tested (Table 1). The generic apicomplexan PCR primers PF1 5′-GCG CTA CCT GGT TGA TCC TGC C-3′ and SSUR4 5′-GAT CCT TCT GCA GGT TCA CCT AC-3′ (Wakeman 2013) and the PCR reaction and cycling conditions described above were used to confirm the presence of apicomplexan DNA.

To identify PCR inhibitors in the 4 DNA extracts that were APX PCR-negative but positive for APX infection by histology, 2 µl template DNA (150 ng μl^{-1}) was mixed with 2 µl salmon DNA (100 µg ml^{-1}). This mixture was then amplified in a 10 µl real-time quantitative PCR (qPCR) prepared using Express qPCR Super Mix (Invitrogen), 1 µl 10 mg ml^{-1} PCR primers Sketa F3 (5′-GGT TTC CGC AGC TGG G-3′) and Sketa R2 (5′-CCG AGC CGT CCT GGT CTA-3′), 0.2 µl hydrolysis probe (RoxIB®RQ 5′-AGT CGC AGG CGG CCA CCG T-3′) and 3.8 µl sterile distilled water. DNA was amplified using a Rotor-Gene Q thermal cycler (QIAGEN) and the thermal cycling conditions 50°C for 2 min, 95°C for 2 min and 40 cycles of 95°C for 15 s, 60°C for 1 min. Quantification cycle numbers were calculated by measuring fluorescence at 610 nm after excitation at 585 nm. Salmon DNA (2 µl) and water were amplified as positive and no-template controls, respectively. To see if the effect of inhibitors in the 4 DNA extracts could be mitigated, PCRs for APX were repeated using DNA templates (150 ng μl^{-1}) diluted to 1:10, 1:30, 1:50 and 1:100 with sterile distilled water respectively (Schrader et al. 2012), and by adding bovine serum albumin (BSA) to each PCR at the final concentration of 0.4 µg μl^{-1} (Carnegie et al. 2006).

RESULTS

PCR test sensitivity and specificity

As determined from gel analyses of the 723 bp DNA product generated using serial 10-fold dilutions

Table 1. Apicomplexan species included in this study to test the specificity of PCR primers developed to detect apicomplexan-X (APX). (+) amplification; (–) no amplification detected. The APX specific primer set APX-For/APX-Rev amplified a 723 bp portion of the APX 18S rRNA gene, the apicomplexan generic primer set PF1/SSUR4 amplified a 1795 bp portion of the apicomplexan 18S rRNA gene. ADHBC: Auckland District Health Board collection (New Zealand); MUC: Massey University collection (New Zealand); HKUC: Hokkaido University collection (Japan)

Species	Source	APX-For/ APX-Rev	PF1/ SSUR4
Toxoplasma gondii	Patient - ADHBC	–	+
Toxoplasma gondii	Vaccine Toxovax® - MUC	–	+
Neospora caninum	Pure culture - MUC	–	+
Selenidium sp.1	Sabellidae - HKUC	–	+
Selenidium sp.2	Sabellidae - HKUC	–	+
Selenidium sp.3	Cirratulidae - HKUC	–	+
Selenidium sp.4	Terebellidae - HKUC	–	+
Cephaloidophorida sp.1	Crustacea - HKUC	–	+
Cephaloidophorida sp.2	Crustacea - HKUC	–	+
Cephaloidophorida sp.3	Crustacea - HKUC	–	+
Lecudina sp.1	Nereididae - HKUC	–	+
Lecudina sp.2	Nereididae - HKUC	–	+
Lecudina sp.3	Nereididae - HKUC	–	+
Lecudina sp.4	Nereididae - HKUC	–	+
Lecudina sp.5	Nereididae - HKUC	–	+
Thiriotia sp.	Crustacea - HKUC	–	+
APX	Flat oysters	+	+

of purified PCR product, the APX 18S rRNA gene PCR test reliably detected down to 0.1 fg DNA (Fig. 2, lane 11), corresponding to 135 gene copies. Of the 75 DNA extracts from frozen flat oyster digestive gland and gill tissue samples, 723 bp DNA products were amplified from 41/41 Grade 1, 23/23 Grade 2, 5/9 Grade 1 and 0/2 Grade 0 (APX-negative) oysters as graded by histology (Hine 2002a). Real-time PCR testing the 4 DNA extracts that were APX PCR-negative after adding salmon DNA using the salmon-specific Sketa F3:R2 primer pair indicated the presence of PCR inhibitors (data not shown). While fluorescence was clearly generated after ~25 cycles when salmon DNA was amplified alone, when mixed with any of the 4 PCR-negative oyster samples scored as Grade 1 for APX by histology, no fluorescence was evident up to 40 cycles. Neither further dilution of the 4 DNA templates nor adding BSA to the PCR tests resulted in detectable fluorescence.

The APX PCR test did not amplify a DNA product using DNA extracted from any of the 16 apicomplexan species tested that spanned 6 genera (Table 1, data not shown), thus supporting the specificity of the APX 18S rRNA test. In addition, purified APX PCR products from positive samples were invariant compared to the APX 18S rRNA gene sequence deposited in GenBank for which the PCR primers were designed (KX774501; Suong et al. 2017).

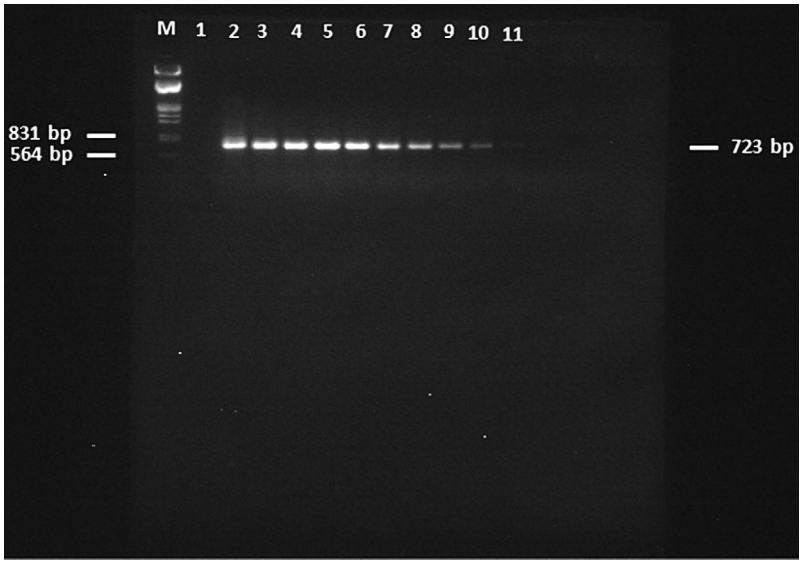

Fig. 2. Detection limit of the PCR primer pairs APX-For/APX-Rev for detection of apicomplexan-X (APX). Lanes: M: PCR ladder (564–21226 bp, Thermo Scientific™); 1: negative control (water); 2 to 13: serial 10-fold dilutions of APX DNA ranging from 100 ng μl^{-1} to 1 ag μl^{-1}. Numbers on the left indicate sizes of molecular markers; the number on the right indicates the size of the amplified product

DISCUSSION

To date, APX detection has relied on laborious methods such as histology using hematoxylin and eosin and periodic acid-Schiff stains, or transmission electron microscopy (Hine 2002a). Such methods are also less informative when a specimen has died and is decomposing or has been frozen. Another drawback is their potential to misidentify morphologically similar apicomplexan species such as the apicomplexans detected in green-lipped mussel (Diggles et al. 2002), Pacific oysters and blue mussels (S. C. Webb unpubl. data).

To address these diagnostic problems, a PCR test specific to the APX 18S rRNA gene was designed and assessed. Test analytical specificity was promoted by designing the PCR primers to 18S rRNA gene sequences differing substantially, particularly at their 3′-ends, from cognate sequences of both oysters and *Bonamia* spp. (Balboa et al. 2011), and by including C and G residues at the primer 3′-ends to further destabilise interactions with mismatched nucleotides (Benita et al. 2003, Balboa et al. 2011, Lorenz 2012).

The test detection limit was 135 APX gene copies μl^{-1} primer, and PCR data correlated well with histological grading of APX infection loads in clinical samples (Hine 2002a). Of the 4/9 Grade 1 clinical samples that tested PCR-negative for APX, qPCR testing for salmon DNA in the presence of oyster DNA identified the co-extraction of PCR inhibitors to be a likely cause of the APX PCR test failing to detect the low concentrations of APX DNA in these samples. Such false-negatives due to PCR inhibitors in DNA extracts of shellfish such as oysters have been associated with high concentrations of polysaccharides such as glycogen in some tissue types (Atmar et al. 1995, Schrader et al. 2012), and with bivalve molluscs may also arise through filter-feeding seston (Schrader et al. 2012). Since dilution of DNA template or addition of BSA to the PCR test did not improve the analytical sensitivity, further work to identify the inhibitory substances and exclude them during DNA extraction might help enhance the PCR test robustness in detecting APX in low-grade infections.

Described here is the first PCR test to specifically detect the apicomplexan-X (APX) parasite found in flat oysters in New Zealand. Benefits anticipated from use of the test include increased speed of detection as well as the ability to confirm histology-based diagnosis, characterise genetic variants and potentially differentiate APX zoites within and among bivalve hosts. The PCR test should also assist in finding the thus-far unrecognised non-zoite life stages of APX, if such stages exist.

Acknowledgements. This research was supported by the New Zealand and Viet Nam governments through a New Zealand-ASEAN Scholarship awarded to N.T.S., and in part by a NZ Ministry of Business, Innovation and Employment grant (Grant Number: CAWX1315) and a New Zealand–Japan Joint Research Programme (Grant Number: JSP-CAW1601-JR). We thank Dr. Stephen Feist and the reviewers for their substantial contribution that greatly improved the quality of the paper. Our thanks also go to Associate Professor Wendi Roe and Ms. Laryssa Howe (Institute of Veterinary, Animal and Biomedical Sciences, Massey University), and Ms. Kitty Croxson and Mr. Arthur Morris (Auckland District Health Board) for helping source apicomplexan DNA. The authors have no conflicts of interest to declare.

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Editorial responsibility: Stephen Feist, Weymouth, UK

Submitted: October 16, 2017; Accepted: May 10, 2018
Proofs received from author(s): July 1, 2018