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Development of a quantitative PCR for rapid and sensitive diagnosis of an intranuclear coccidian parasite in Testudines (TINC), and detection in the critically endangered Arakan forest turtle (*Heosemys depressa*)

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ABSTRACT

The intranuclear coccidian parasite of Testudines (TINC) is responsible for significant disease in turtles and tortoises causing high mortality and affecting several threatened species. Diagnostic testing has been limited to relatively labor intensive and expensive pan-coccidial PCR and sequencing techniques. A qPCR assay targeting a specific and conserved region of TINC 18S rRNA was designed. The qPCR reaction was run on samples known to be TINC positive and the results were consistent and analytically specific. The assay was able to detect as little as 10 copies of target DNA in a sample. Testing of soil and invertebrates was negative and did not provide any further insights into life cycles. This assay was used to identify TINC in a novel host species, the critically endangered Arakan forest turtle (*Heosemys depressa*).

1. Introduction

Chelonians have been found to be hosts for more than 30 different species of coccidian parasites. Of these, *Caryospora cheloniae* infection in green turtles (*Chelonia mydas*), and the novel tortoise intranuclear coccidian species (TINC) described by Jacobson et al. (1994) have been reported to cause significant pathologic changes. The parasitic coccidia of vertebrates most commonly have endozoic development (merogeny and gamogeny) within the cytoplasm of enterocytes, although at least 11 other species of *Eimeria*, *Isospora*, and *Cyclospora* are reported to be caryotropic (having intranuclear life stages) (Garner et al., 2006). TINC are found in cell nuclei of numerous organs, including the GI tract, pancreas, liver, kidney, and spleen (Garner et al., 2006). In Sulawesi tortoises (*Indotestudo forsteni*), this organism has been associated with erosive rhinitis (Innis et al., 2007). Mortality in identified cases appears to be high, as 29 of 41 flat-tailed tortoises (*Pyxis planicauda*) (71%) died over a 2 yr period in one zoological collection (Praschag et al., 2010). Clinical signs include lethargy, rapid weight loss, weakness, gasping respiration, and swollen erythematous vents with gross evidence of epidermal necrosis. These signs are not specific and vary among individuals. The life cycle of this organism is not known. Although a genus has not been assigned to this organism, its phylogenetic position is fairly well characterized by sequencing of the 18S small ribosomal subunit gene (Innis et al., 2007).

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Since the discovery of TINC, there has been an increasing number of animals with systemic illness found to have lesions on necropsy. Species reported to be affected by TINC have included the radiated tortoise (*Astrochelys* [*Geochelone*] *radiata*), impressed tortoise (*Manouria impressa*), leopard tortoise (*Psammobates* [*Geochelone*, *Stigmochelys*] *pardalis*), bowsprit tortoise (*Chersina angulata*), and Sulawesi tortoise (*I. forsteni*) (Garner et al., 2006; Innis et al., 2007). Our laboratory has also identified this organism in a Galapagos tortoise (*Chelonoidis* [*Geochelone*] *nigra becki*), Indian star tortoise (*Geochelone elegans*), eastern box turtle (*Terrapene carolina carolina*), flat-tailed tortoise (*Pyxis planicauda*), and spider tortoise (*Pyxis arachnoides*). TINC has been found in captive tortoises in both North America and Europe (Garner et al., 2006; Innis et al., 2007; Schmidt et al., 2008).

The Arakan forest turtle is a critically endangered turtle native to Myanmar (Asian Turtle Trade Working Group, 2000). First described in 1875, only 5 animals were reported until 1908, and the species was then unreported until 1997 (Platt et al., 2010). Diseases in this species are poorly understood; there is a report of an upper alimentary tract disorder of unknown etiology (Innis et al., 2006), and a description of *Eimeria mitraria* and *Eimeria arakanensis* (Široký and Modrý, 2006).

Currently, the only test providing a definitive diagnosis of the presence of TINC is pan-coccidial PCR and sequencing (Garner et al., 2006). This entails DNA extraction from the sample, polymerase chain reaction using primers designed to amplify any coccidian species present, gel electrophoresis to separate and evaluate PCR products, extraction of bands of appropriate size from the agarose gel, running cycle sequencing of the extracted band, running a sequencing gel, and analysis of sequence data. This process, while providing a definitive identification of any coccidial organism, is labor-intensive, relatively expensive, and usually takes several days.

In contrast, quantitative PCR (qPCR, real-time PCR) is a methodology that uses hybridization of a sequence-specific probe during the PCR reaction to validate product identity. As the polymerase advances past the hybridized probe, exonuclease activity digests the probe and labeled dyes are released. The released dyes are spectrophotometrically measured. Specific product synthesis can therefore be determined as the reaction progresses, providing not only qualitative information regarding presence, but also quantitative information. The steps are significantly less labor intensive, entailing DNA extraction from the sample, qPCR, and data analysis. qPCR assays have been used successfully for coccidian testing (Vrba et al., 2010), and are often found to be more sensitive than standard PCR (Vidal et al., 2011).

This simpler, less expensive test allows for cost-effective cross-sectional study of collections that contain potentially susceptible animals. Such screening may help reduce mortality in assurance colonies of the threatened and endangered species affected by TINC. The aim of this study was to develop a quantitative PCR (real-time or qPCR) to diagnose the presence of TINC. This assay will be more rapid and less expensive than the only currently available definitive diagnostic test, and will provide quantitative information. The development of this assay will enable studies optimizing diagnostic sampling and investigating life cycles.

2. Materials and methods

2.1. Samples

Samples examined for TINC included eleven diagnostic samples from chelonians previously submitted to our laboratory that were positive for TINC using consensus PCR and sequencing (Garner et al., 2006). To determine the specificity of the assay, DNA preparations of additional coccidian species were used including *Eimeria gruis* from a hooded crane (*Grus monacha*) sample, *Eimeria southwelli* from a cownose ray (*Rhinoptera bonasus*) sample, an *Eimeria* sp. from a Bali mynah (*Leucopsar rothschildi*) sample, and *Cryptosporidium baileyi* from a princess parrot (*Polytelis alexandrae*) sample. Nine additional clinical samples from apparently healthy chelonians were tested (Table 1). Eight environmental samples from an enclosure that had contained tortoises where fatal TINC cases had occurred were also tested (Table 1). These environmental samples included soil samples, as well as invertebrates found in the enclosure.

2.2. Amplification and sequencing, and gene identification

The 18S rRNA gene was amplified by PCR using a hemi-nested protocol. The hemi-nested PCR used primers 18F (5′-CTGGTTGATCCTGCCAGTAGTC-3′, Innis et al., 2007) and 1503R (5′-CYTCCYTRCRTTARACACGCAA-3′, Garner et al., 2006) in the first round, and primers 18F and INC-qPCR2R (5′-AACCCGCACAGTGAAGTGG-3′) in the second round. Amplifications were performed using Takara SpeedStar HS DNA Polymerase (Takara Bio Inc., Otsu, Japan) as follows: 5 min denaturation at 94 °C, followed by 40 cycles of denaturation at 94 °C (30 s), annealing at 48 °C (60 s), extension at 72 °C (120 s), with a final elongation step at 72 °C for 7 min. PCR products were run in 1% agarose gels. Bands of interest were cut from the gel and DNA was extracted using the Qiaquick gel extraction kit (Qiagen, Valencia, CA). Direct sequencing was performed using the Big-Dye Terminator Kit (Applied Biosystems, Foster City, CA) and analyzed on an ABI 3130 automated DNA sequencer at the University of Florida Interdisciplinary Center for Biotechnology Research Sequencing Facilities.

2.3. Quantitative PCR

Quantitative PCR was performed on samples using forward primer INCqPCRF (5′-GTTTGGTCCGCCTCCTTG-3′), reverse primer INCqPCR2R, and a probe targeting the intranuclear coccidian organism conserved region (5′-6FAM-GCCGGTCGAACTTCAGCATC-BHQ-3′). The region was designed to be specific by comparison to other aligned coccidian 18S rRNA sequences and by using BLASTN (Altschul et al., 1997). Each 20 μL reaction was run in duplicate, and consisted of 18 μM for each primer and 5 μM for the probe, and contained 7 μL of extracted DNA and 10 μL of a commercial universal qPCR mix (TaqMan® Fast

Table 1
Samples tested and qPCR results.

Sample	Host	Sample	Consensus PCR/sequencing result	qPCR result	Mean TINC[a] quantity via qPCR	Standard deviation	18S Ct[b]
1	*Polytelis alexandrae*	Feces	*Cryptosporidium baileyi*	Neg	Undetected	–	19.86
2	*Leucopsar rothschildi*	Whole blood	*Eimeria* sp.	Neg	Undetected	–	15.8
3	*Grus monacha*	Buffy coat	*Eimeria gruis*	Neg	Undetected	–	18.42
4	*Rhinoptera bonasus*	Coelomic fluid	*Eimeria southwelli*	Neg	Undetected	–	19
5	*Terrapene carolina carolina*	Paraffin-embedded tissue	TINC	Pos	52,713	7946	42.53
6	*Pyxis* spp.	Choanal swab	TINC	Pos	79,241	7712	16.15
7	*Pyxis* spp.	Oral/cloacal swab	TINC	Pos	96,165	17,032	32.97
8	*Pyxis* spp.	Oral/cloacal swab	TINC	Pos	186,020	6704	16.37
9	*Pyxis* spp.	Oral/cloacal swab	TINC	Pos	397,052	62,882	18.43
10	*Pyxis* spp.	Choanal swab	TINC	Pos	390	128	38.95
11	*Pyxis* spp.	Swab[c]	TINC	Pos	25,093	6521	39.82
12	*Geochelone nigra becki*	Swab[c]	TINC	Pos	7785	330	15.5
13	*Pyxis* sp.	Swab[c]	TINC	Neg	Undetected	–	Undetected
14	*Pyxis* sp.	Swab[c]	TINC	Neg	Undetected	–	Undetected
15	*Pyxis arachnoides brygooi*	Swab[c]	TINC	Neg	Undetected	–	Undetected
16	*Heosemys depressa*	Cloacal swab	TINC	Pos	35,656	1897	22.93
17	*Heosemys depressa*	Cloacal swab	TINC	Pos	36,664	1379	22.28
18	*Heosemys depressa*	Cloacal swab	TINC	Pos	132,030	7127	39.23
19	*Manouria emys phayrei*	Cloacal swab	Not tested	Neg	Undetected	–	Undetected
20	*Manouria impressa*	Cloacal swab	Not tested	Neg	Undetected	–	23.24
21	*Manouria impressa*	Cloacal swab	Not tested	Neg	Undetected	–	41.7
22	*Manouria impressa*	Cloacal swab	Not tested	Neg	Undetected	–	25.53
23	*Manouria impressa*	Cloacal swab	Not tested	Neg	Undetected	–	29.46
24	*Terrapene carolina Mexicana*	Cloacal swab	Not tested	Neg	Undetected	–	43.2
25	Enclosure	Slug	Not tested	Neg	Undetected	–	20.45
26	Enclosure	Earwig	Not tested	Neg	Undetected	–	16.6
27	Enclosure	Centipede	Not tested	Neg	Undetected	–	16.05
28	Enclosure	Sowbugs	Not tested	Neg	Undetected	–	Undetected
29	Enclosure	Sowbugs	Not tested	Neg	Undetected	–	Undetected
30	Enclosure	Soil	Not tested	Neg	Undetected	–	Undetected
31	Enclosure	Soil	Not tested	Neg	Undetected	–	Undetected
32	Enclosure	Soil	Not tested	Neg	Undetected	–	Undetected

Note: Samples 13–15 did not amplify either host or TINC 18S, indicating sample degradation.

[a] Tortoise intranuclear coccidian parasite.

[b] Crossover threshold – the cycle number at which the cutoff fluorescence is reached.

[c] Samples listed in the table as only "swab" were listed as such because these samples were previously banked and more information regarding the origin of the swab sample was not recorded at the time of collection. These samples were either choanal or cloacal swabs.

Universal PCR Master Mix 2X, Applied Biosystems), using a standard fast protocol.

The standard curve, run on each plate, used a 10-fold serial dilution of the intranuclear coccidian organism PCR amplicon from the index case, because culture of the organism is not currently possible. The template was quantified by both spectrophotometry and comparison to gel standards. Dilutions were made, and control standards were run using 10^1–10^6 copies in duplicate on each plate. Appropriate function of qPCR probes and primers was assessed by evaluating R^2 values and slopes of the standard curves. A Eukaryotic 18S rRNA Endogenous Control kit (VIC/MGB

Probe, Applied Biosystems, Foster City, CA) was used to validate the presence of amplifiable DNA in each sample in a separate well, and samples that failed to amplify 18S were rejected. A 7500 Fast Real-Time PCR System (Applied Biosystems) was used to amplify the reactions with cycling conditions as follows: initial denaturation at 95 °C for 20 s; 50 cycles of 95 °C for 3 s followed by 60 °C for 30 s. Each sample was tested in duplicate on each plate as well as an 18S control. The samples were tested twice. The software included with the ABI 7500 fast equipment was used to calculate slopes and R^2 values.

3. Results

3.1. Estimation of the number of copies in a given sample

The standard curve generated by 10-fold serial dilutions of DNA was found to be linear, with a slope of 3.86, indicating 81.6% efficiency and an R^2 value of 0.998. Samples run on the qPCR are compared to the standard curve to estimate the number of copies present, and results are given in Table 1.

3.2. Specificity of qPCR assay

Of the samples previously tested by pan-coccidial consensus PCR and sequencing, only known TINC positive samples reacted and were confirmed positive by the qPCR assay. All other related and unrelated nontarget DNA preparations were negative (Table 1). Three archived previously TINC positive samples (Table 1: samples 13–15) did not amplify either host or TINC 18S, indicating sample degradation, and were rejected. Both the positive and negative test results were consistent each time the samples were tested.

3.3. Additional tortoise and environmental samples

The nine additional clinical samples of apparently healthy chelonians tested from a zoological collection tested negative, with the exception of samples from Arakan forest turtles (*Heosemys depressa*). The eight environmental samples tested from an enclosure that contained TINC positive tortoises all tested negative (Table 1). The samples including sowbugs did not register in the 18S eukaryotic control well.

4. Discussion

With the development of this qPCR assay, the presence of TINC in turtles and tortoises can be identified in a single day rather than the several days that the previously available testing required, and quantitative information is now available. Reagent and personnel time costs are reduced. The assay can now be used to elucidate many important unknown factors regarding this infectious agent.

The efficiency of this qPCR assay was found to be relatively low (81.6%), indicating that there was less than a complete doubling of product every cycle. Common reasons for lower efficiency include the presence of PCR inhibitors and steric issues with primers/probes. Because purified PCR product was used in the standard curve, we consider the presence of PCR inhibitors unlikely. The target region was chosen due to the divergence of this region from other coccidia in an otherwise highly conserved gene. The linearity of the standard curves was excellent, with R^2 values over 99%, and we were able to detect low copy numbers reliably with this assay.

The assay was able to detect as little as 10 copies of target DNA in a sample. While the specificity and sensitivity of this assay, as compared to the consensus PCR and sequencing assay previously utilized, both appear to be 100%, this should be interpreted with some caution, due to the small sample size as well as the potential limitations of the use of the consensus PCR and sequencing assay as a gold standard.

The life cycle and ecology of the intranuclear coccidian organism of Testudines are unknown, which is the primary reason this organism has not been named. Life cycles vary among coccidian species and may involve vector transmission, fecal transmission, and intermediate hosts. Both sexual and asexual reproduction may occur at various life cycle stages. TINC has shown several typical apicomplexan life stages, including oocysts, macrogametes, microgametes, meronts, merozoites, and trophozoites (Innis et al., 2007). Other than these findings on histopathology, much of the life cycle is unknown. This assay provides a tool to help uncover some of these details by testing environmental samples including possible intermediate hosts in the area of affected animals for the intranuclear coccidian organism, prompting a further investigation in any samples that test positive. The environmental samples tested by our laboratory represented the first effort in exploring possible intermediate hosts and environmental burdens of the parasite. No environmental samples tested positive for the presence of TINC. Both samples including sow bugs did not amplify the eukaryotic 18S control. This may have been due to natural PCR inhibitors in these isopods or primers mismatching. A definitive cause cannot be determined based on our data. All reported cases of TINC to date have been in captive animals. Those captive tortoises have not been reevaluated for the presence of the organism since the initial diagnosis. Serial quantitative information from an infected individual could aid in describing the course of the disease and response to treatment.

Currently, only animals in zoological collections in the U.S. and Austria have been reported to be infected with TINC. However, testing in other countries has not been reported. Because animals are occasionally transferred among zoological facilities, even to other countries, having a test that allows for economical and rapid diagnosis of TINC is a valuable tool to prevent inadvertent spread of the organism to naïve sites. Additionally, no work has been done yet to survey the prevalence of the organism or the disease in wild populations. This assay provides a cost effective way to test free-ranging tortoises and provide data regarding its distribution in wild populations of tortoise as well as the prevalence in zoological collections worldwide. Of the nine additional clinical samples of chelonians tested from a zoological collection, only samples from

Arakan forest turtles (*H. depressa*) were found to be positive. The assay was used as a screening tool of apparently healthy turtles with a history of exposure to a tortoise that died of TINC. Positive results in apparently healthy individuals merit further investigation. This is the first time TINC has been reported in *H. depressa*, an IUCN red list critically endangered species (Asian Turtle Trade Working Group, 2000).

We note the lack of diversity seen in TINC sequences from diverse host species to date. While it is possible that outbreaks to date may have been clonal, the only sequence data available from TINC so far is from rRNA genes. These are the most conserved genes present in any cellular organism; further investigation of additional less conserved genes would be necessary to look for variation.

The distribution of organisms in an infected tortoise has only been described via histopathology. This assay can quantify the numbers of copies present in samples to aid in describing the organismal burden in different tissues.

It should be noted that our qPCR values are for copies of target DNA detected, which may potentially differ from actual copy numbers and organisms present. We are unable to culture TINC, and thus we are unable to test known numbers of whole organism samples directly. We used dilutions of known copy numbers of TINC DNA as a standard curve. This control is a DNA template and does not reflect loss during extraction. The presence of PCR inhibitors or nucleases may result in falsely low readings. These are common in feces, so this is of special concern for fecal samples (Monteiro et al., 1997). Control 18S rRNA amplification is often variable in fecal samples, and so equivalency is based solely on use of an equivalent amount of feces used for initial extraction. Further, extraction efficiency may differ between tissue samples and fecal samples, so caution should be used to avoid over-interpretation when comparing different sample types (Verheyen et al., 2012).

5. Conclusion

In this report, we describe the development of a qPCR assay that provides a quick and analytically specific method for screening TINC, a pathogen found in a variety of tortoise species, including some that are critically endangered species. We hope this qPCR assay will be of use for further surveillance of this disease, disease distribution, description of the organism's life cycle, and possibly response to therapy.

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