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Development of reverse-transcriptase quantitative PCR assays for detection of the cytokines IL-1β, TNF-α, and IL-10 in chelonians

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ABSTRACT

In response to viral pathogens, a host releases pro-inflammatory cytokines such as interleukin-1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α) and anti-inflammatory cytokines such as interleukin-10 (IL-10). While several approaches exist to measure cytokine responses, evaluating gene transcription through reverse transcription quantitative polymerase chain reaction (RT-qPCR) provides a fast, reproducible, and sensitive method for quantifying this response. The objective of this study was to develop an effective and sensitive RT-qPCR assay for the quantification of red-eared slider (*Trachemys scripta elegans*) and eastern box turtle (*Terrapene carolina carolina*) cytokines: IL-1β, TNFα, IL-10 and the reference gene β-actin. RNA was isolated from the buffy coat layer of whole blood, comprised mainly of circulating leukocytes, and complimentary DNA (cDNA) was produced. Conventional PCR was performed to obtain cytokine mRNA sequences, products were sequenced, and a hydrolysis probe-based RT-qPCR assay was designed for each cytokine. Standard curves were generated using the target gene sequences cloned within a plasmid. Efficiencies for each assay were between of 85–110%, $R^2 > 0.98$, and limits of detection of 10–100 copies per reaction. The initial samples used to identify the novel target sequences were then used to evaluate the performance of the qPCR assays. Consistent transcription of beta actin across individuals in both species and measurable transcription of IL-1β, TNF-α, and IL-10 transcript targets in individuals of both species were observed. The assays are a novel technique in chelonians to evaluate host innate immune response.

1. Introduction

Host-pathogen interactions comprise a major component in understanding and evaluating the health of individuals and populations [8,12,2,22,1]. The success of the host response in the short and long term determines the outcome when interacting with a pathogen [26]. Immune responses can be influenced by many factors, including individual variation, temporal relationships, and potential pathogen interference [18;11,26,24]. As such, the evaluation of immune response can offer insight to host mechanisms and potential outcomes of interest, specifically morbidity and mortality, at the individual and population levels.

Methods of measuring immune response include detecting markers for inflammation, such as acute phase proteins, the humoral response (antibodies), and relative genetic transcription of cytokine mRNA [34,14;3]. The advantages and limitations of each method drive their use among fields of interest. The development of cytokine mRNA transcription assays is a popular approach for species-specific immune response evaluations within the field of wildlife epidemiology [1].

Cytokines are transient signaling proteins that stimulate cellular responses locally and systemically in response to inflammatory stimuli [37]. As a non-specific host response, inflammation can occur relative to varied stimuli. However, the combination of timing and comparison with other health parameters (hematology, viral load) can lead to a better understanding of host immune activity. While direct measurement would best indicate real-time host response, the transient nature of these molecules makes detection very difficult [3]. The lack of sensitive, species-specific protein assays as well as specific timing protocols for sampling present significant obstacles for analysis of cytokines in non-model species. As such, cytokine mRNA transcription quantification is commonly used, and previous studies have found that that an increase in transcription for cytokine molecules correlates with an increased cytokine presence and inflammatory response [17,29]. Due to the conserved nature of cytokines, these types of assays have been

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developed and used to quantify cytokine mRNA transcription across varied species, as they have in mammals such as ferrets [27,7], elephants [20], manatees [9], and in bird species [16].

Cytokine transcription analyses in ectotherms have been performed *in vitro* using fathead minnow (*Pimephales promelas*), African small-clawed frog (*Xenopus laevis*), and Chinese soft-shelled turtle cultured cells (*Pelodiscus sinensis*)[36;23;15;10;35]. In these studies, transcription of inflammatory cytokines such as interleukin 1 beta (IL-1β) and tumor necrosis factor alpha (TNFα) increased in response to ranavirus infection and lipopolysaccharide (LPS) stimulation. For the anti-inflammatory cytokine interleukin 10 (IL-10), transcription was significantly greater in the challenged cells 1 h post-infection and significantly less in the challenged cells at 72 h post-infection [15]. This pattern suggests an attempt to mediate early inflammation, followed by suppressed anti-inflammatory action in the infected cells after 72 h. FV3 gene transcription may be interfering with the host IL-10 transcription [11]. Taken together, these *in vitro* studies provide patterns of increased inflammatory cytokine transcription and alternating levels of anti-inflammatory cytokine transcription post-infection for model systems *in vivo* among ectotherms.

Chelonians are one of the most imperiled vertebrate groups [25]. According to the International Union for the Conservation of Nature (IUCN), 148 out of 356 recognized modern species (41.6%) are officially regarded as globally Threatened, which include statuses of Critically Endangered, Endangered, or Vulnerable [28]. Generating data on the varied immune response is a valuable tool for individual and population assessments. The red-eared slider (*Trachemys scripta elegans*), an abundant species that ranges from southeast Virginia to Florida west to New Mexico and south to Brazil, is a primarily aquatic pond turtle in the family Emydidae [4]. It is considered invasive in several locations (IUCN), especially in Europe, Asia, and non-native regions of North America [31]. Red-eared sliders have been identified as useful models for ranaviral disease progression [19]. The eastern box turtle (*Terrapene carolina carolina*) is a primarily terrestrial species that ranges from Maine to Georgia and west to Michigan, Illinois, and Tennessee [4]. According to the IUCN, eastern box turtles are listed as vulnerable, with the apparent high sensitivity of this species to ranavirus as a contributing factor [30]. With low fecundity, low juvenile survival rates, and long adult life spans, eastern box turtles have a life history that predisposes them to impaired population recovery following the loss of adult animals (such as loss by disease) [13].

Our objective was to develop a series of RT-qPCR assays targeting cytokine mRNA in two species of chelonians that may be used to characterize host response to disease. Our hypothesis was that RT-qPCR assays would be sensitive and specific for detection of IL-1β, TNFα, and IL-10 in the red-eared slider and the eastern box turtle. We expected that β-actin would be an appropriate reference gene, acting as an endogenous control to standardize target cytokine genes across cell types and host conditions to allow the comparison of target cytokine transcription ratios. Sampling methodologies were applied in laboratory and field settings to observe practical applications and results. Ultimately, the cytokine assays will be used as a tool to better measure chelonian host immune response to current and future disease threats.

2. Materials and methods

2.1. Sample selection and preparation

Guidelines for RT-qPCR validation were consulted for the development of the cytokine assays (MIQE Guidelines [6,5]. All protocols were assessed and approved under University of Illinois Institutional Animal Care and Use Committee (Protocols #15225 and #18000). Samples were selected from animals that were part of other studies and had a high likelihood of expressing the representative level of inflammation to detect novel gene sequences. A total of 81 RNA samples from twenty-four red-eared sliders (RES, 67 samples [45 pre-infection, 22 post-infection]) and nine eastern box turtles (EBT, 14 samples) were utilized during hydrolysis primer-probe development to test the identification of cytokine mRNA transcripts. All captive RES were females housed at either 28 °C or 22 °C that were part of a study to investigate the pathogenesis of challenge with FV3-like ranavirus. Samples initially screened included two time points prior to inoculation (45 samples from 24 individuals) and one day following inoculation on the suspected peak day for cytokine mRNA transcription (22 samples). The time points were chosen for differential immune response, with the general goal of higher anti-inflammatory cytokine transcription levels prior to infection and increased inflammatory cytokine transcription post-inoculation. In a separate field study, apparently healthy free-range EBT RNA samples (N = 14) were initially screened from two sampling days in the spring sampling period (May) from five adult males (N = 6) and four adult females (N = 8). In both species, approximately 400 μL of whole blood were drawn from the subcarapacial sinus, placed in a lithium heparin microtainer (Becton-Dickinson), and then stored on ice until analysis. Approximately 1 h passed on ice after RES sample collection before processing, and EBT samples were on ice up to six hours in a cooler while in the field. Once in the laboratory, plastic hematocrit tubes (Drummond Scientific Company, Broomall, PA) were loaded with 50 μL of heparinized whole blood and centrifuged at 10,000 rpm X 5 min. The microhematocrit tubes were cut with a sterile blade to isolate the buffy coat of the separated cells using a previously described method [33]. The entire plastic portion of tube was placed in approximately 60 μL of RNALater (Ambion Inc., Foster City, CA) and stored in a −20 °C freezer until RNA extraction.

2.2. RNA extraction and cDNA production

RNA was extracted using a commercial kit according to the manufacturer's recommendations using RNA-only lab equipment (Qiagen RNEasy, Valencia, CA). After extraction, the RNA samples were analyzed using spectrophotometry (NanoDrop 1000, Thermo Fisher) to check for quantity and an overview of purity. The RNA was stored in a −80 °C freezer until reverse transcription into cDNA, which was performed using the Quantitect Reverse Transcription kit using a mix of oligo-dT primers and random primers after using the genomic DNA elimination buffer (Qiagen, Valencia, CA). A constant volume of 2 μL of RNA template were used for each reaction in batches (N = 50), and samples were incubated for 15 min at 42 °C according to the protocol. All cDNA products were analyzed using spectrophotometry and stored at −20 °C until dilution with sterilized (autoclaved) dH_20 at 1:40 vol yielding approximately 50 ng/μL, for further analysis.

2.3. Selection of primers and probes

Degenerate primers were designed for the cytokine mRNA targets of TNFα, IL-1β, and IL-10 using the codon-equivalent multiple alignment software, CEMASuite [21]. Briefly, amino acid sequences were obtained and aligned for multiple chelonian species including a painted turtle (*Chrysemys picta*), a green sea turtle (*Chelonia mydas*), a Chinese soft-shelled turtle (*Pelodiscus sinensis*), and a vertebrate outgroup when possible (Table 1). Multiple potential nucleotides at a single base pair location were coded as degenerate, and primers were synthesized.

Conventional PCR targeting the gene regions identified *in silico* used the following reagents per reaction: 37.75 μL sterile dH_2O, 5.0 μL 10x PCR Buffer, 3.0 μL $MgCl_2$, 1.0 μL dNTP, 0.25 μL *Taq* polymerase, 1.0 μL forward primer (10 pmol), and 1.0 μL reverse primer (10 pmol). PCR protocols were run on a C1000 Thermal Cycler (Bio-Rad Laboratories Inc., Hercules, CA). Multiple annealing temperatures (T_A) were used based on the primer qualities starting at approximately 5 °C below the provided melting temperature (T_M). The base protocol to test the primers was: 3 min 95 °C, 35 cycles of: 30 s 95 °C, 30 s T_A°C, 1 min 72 °C; 5 min 72 °C, and the final hold stage at 4 °C.

PCR products were resolved on a 1% agarose gel with ethidium

Table 1
Conventional primers designed using the codon-equivalent multiple alignment method with the CEMASuite software [21]. Vertebrate outgroups were used when possible to find the most-conserved regions.

Cytokine	Species (NCBI accession #)	Primers	Base Pairs
beta actin	*Pelodiscus sinensis* (ACD99706) *Mauremys mutica* (ADX86815) *Trachemys scripta* (ACL50286)	F:GTGCTGTCCCTGTATGCCTC R:GTTTCATGAATGCCACAGGA	416
TNFα	*Pelodiscus sinensis* (XM_014575959.1) *Chelonia mydas* (XM_007053203.1) *Chrysemys picta* (XM_008176809.1)	F:CTGGAAATGYCTSAGCATCTGT R:GACWGCWCCTTGGTAGATGGA	517
IL-1β	*Pelodiscus sinensis* (XM_014571852.1) *Chelonia mydas* (XM_007066028.1) *Chyrsemys picta* (XM_005279260.2)	F: TGCAGTTCACAGAGCAGACC R: GACCGGAAACCTCATGGAG	383
IL-10	*Mus musculus* (NC_000067) *Chelonia mydas* (NW_006649512) *Chrysemys picta* (NW_007281457)	F: GCTCTCTGAAGAAAGCTGCAC R: CACTTTCCCATCTTCATCATCA	468

bromide and observed under UV light. Products were processed using the ExoSap enzyme (Thermo Fisher) and then Sanger sequenced (ACGT, Inc.). The sequences were compared to known sequences in BLAST (NCBI). A single positive sequence was used for each species and cytokine. Hydrolysis probes were then designed using commercial software (Primer Express Software, v3.0.1, Thermo Fisher) targeting the sequences obtained from conventional PCR or the predicted cytokine sequences for *Chrysemys picta* (Table 2).

Table 3
Taqman primer-probe validation for 5 cytokine mRNA targets in chelonians. Numbers within the primer-probe name indicate the starting base pair within the target, testing multiple sets per cytokine sequence. Ideal slope is −3.32 (=100% efficiency), with an acceptable range of 85–110% efficiency.

Name	Slope	Efficiency (%)	R^2	Limit of Detection (# copies)
RES Bac 212	−3.319	100.12	1.000	10
RES Bac 43	−3.324	99.91	0.998	10
Cons Bac EBT	−3.426	95.83	1.000	10
IL1B 16	−3.538	91.71	0.999	100
IL1B 55	−3.314	100.33	0.999	10
TNF	−3.408	96.53	0.998	100
IL10 80	−3.360	98.44	1.000	10
IL10 316	−3.318	100.16	1.000	100

2.4. Construction of cDNA standard

Genes representing cytokines and the reference gene (Table 3) were designed and synthesized (GeneArt Gene Synthesis, Life Technologies). The kanamycin-resistant plasmid was transformed in corresponding resistant bacteria after permeabilization. The bacterial plasmid was purified with QIAfilter Plasmid Maxi Kit (Qiagen, Venlo, Netherlands). Plasmids were linearized using the *Sfil* enzyme kit (Thermo Fisher) according to protocol, and then the DNA was extracted using phenol:-chloroform and ethanol precipitation. Nucleic acid concentration was determined with a spectrophotometer (NanoDrop 1000, Thermo Fisher). Cytokine copy numbers were then calculated using the formula: Number of copies = (DNA quantity * 6.022×10^{23})/(DNA length * 1×10^9 * 650). A 10-fold standard dilution series was generated in RNAse/DNAse free water, and dilutions from 1×10^1 to 1×10^7 copies/μl were employed for subsequent RT-qPCRs.

2.5. Reverse-Transcription quantitative PCR

Reverse-transcription qPCR assays were performed using a real-time PCR thermocycler (7500 ABI RealTime PCR System, Applied

Table 2
Taqman primer-probes designed using PrimerExpress. Numbers within the name indicate the base pair at which the forward primer starts, to differentiate sets for the same cytokine mRNA. NCBI reference numbers are listed below the set names to identify the target transcript. Names marked with * were designed using the reverse complement of the GenBank sequence, and the base pair (bp) position is displayed accordingly. IL1B positions refer to a consensus sequence made using the unique RES and EBT sequences. F = forward, R = reverse, p = probe sequences.

Name (Target)	Primers Probe Sets	Position (bp)
RES Bac 212*	F: TGGCCATCTCCTGTTCGAA	279–261
(MH195268)	R: GGAAATTGTACGTGACATAAAGGAAA	212–237
	P: CCAGAGCAACGTAGCAC	258–242
RES Bac 43*	F: GGGCAGAGCGTAACCTTCATAG	110–189
(MH195268)	R: GTATTGTTATGGACTCTGGTGATGGT	43–68
	P: TGGGCACAGTGTGGG	87–73
Cons Bac EBT*	F: ACCTTCATAGATGGGCACAGTGT	101–79
(MH195269)	R: TGGTCGTACCACAGGTATTGTGA	32–54
	P: ACACCATCACCAGAGTC	73–57
IL1B 16	F: GGTCGCCGTAGAGAAGATGAA	16–36
(RES - MH195270)	R: CCTCAAATCATCGTCCGTGAA	79–59
(EBT - MH195271)	P: AAGGCATCGAAAGTGAG	38–54
IL1B 55	F: GCCCTTCACGGACGATGAT	55–73
(RES - MH195270)	R: TCGAATGAGATGGTCTCGAAGA	117–96
(EBT - MH195271)	P: TGAGGAGCATCTTCGACA	75–92
TNF*	F: CTTTGGGATCCTGGCTGATC	114–95
(MH195272)	R: CTTCGCTCTGCTGCATTTCA	59–78
	P: AGGGCCCCCCGTTT	93–80
IL10 80	F: GCTGCACAAAACTCGCCAAT	80–99
(XM_005306473.1)	R: GCAATCCGCAGGTCTTTGA	134–116
	P: TCCTGCCCCTTCGG	101–114
IL10 316	F: CGGAGCGTGGCCAATATT	316–333
(XM_005306473.1)	R: CGCGTCAGTGTCTGCTTCA	371–353
	P: CATCATGCTGCTGGAC	336–351

Biosystems, Carlsbad, CA) and data were analyzed using associated software (Sequence Detection Software v2.05, Applied Biosystems, Carlsbad, CA). Each 25 μL reaction contained: 12.5 μL TaqMan Platinum PCR Supermix-UDG with Rox (Invitrogen, Carlsbad, CA), 10.0 μL sterile dH_20, 1.25 μL hydrolysis primer-probe, and 1.25 μL sample cDNA. Cycling parameters were as follows: one cycle at 50.0 °C for 2 min, 95.0 °C for 10 min, then 40 cycles of 95 °C for 15 s and 60.0 °C for 1 min.

To determine the analytical sensitivity, assays were performed in three technical repeats of plasmid dilutions ($1 \times 10^7 - 1$ copies/rxn) within a single run. Standard curves were generated using the cycle threshold values of the plasmid dilutions. Intra-assay variation was determined by calculating the mean critical threshold (Ct) values, standard deviations, and coefficient of variations separately for each plasmid dilution. The same values were then calculated to determine inter-assay variation.

After validation, the selected assays were applied to the 81 samples, measuring cytokine mRNA transcription in each sample relative to β-actin transcription. The efficacy of each assay using actual samples was evaluated.

3. Results

3.1. RNA extraction and cDNA production

The buffy coat isolation method and subsequent RNA extraction had a mean yield of 17.69 ng/μl and 260/280 ratio of 1.78. The integrity of the RNA was confirmed by successful downstream applications after cDNA synthesis.

3.2. Selection of primers and probes

PCR products were obtained and partial sequences confirmed for beta actin in a red-eared slider (GenBank #MH195268) and an eastern box turtle (GenBank # MH195269), IL-1β in a red-eared slider (GenBank # MH195270) and an eastern box turtle (GenBank # MH195271), and for TNFα (GenBank # MH195272) in a different red-eared slider. The aligned forward and reverse product sequences were used to design the hydrolysis primer-probes. We were unable to sequence product for IL-10 for either species. Predicted sequences for from NCBI (*Chrysemys picta* XM005306473.1) were used for IL-10 RT-qPCR design. A total of eight primer-probe sets were designed, three for β-actin, two each for IL-1β and IL-10, and one each TNFα.

3.3. Construction of cDNA standard and RT-qPCR

Successful plasmid DNA linearization was confirmed using gel electrophoresis. After ethanol precipitation, the average DNA quantity for the six sequences was 21.55 ng/μL and the average 260/280 ratio was 1.77. These values were appropriate for downstream applications, specifically to create the 10-fold serial dilutions to make the standard curve for each hydrolysis primer-probe test.

Each primer-probe set combination demonstrated acceptable slopes and efficiency (85–110%) and R^2 values (> 0.99), and lower limits of detection between 10 and 100 copies (Fig. 1, Fig. 2, Table 3). Furthermore, comparison of intra-assay and inter-assay critical threshold values for the primer-probe sets were within acceptable limits for the range of quantities tested (Table 4).

The 81 turtle RNA samples provided evidence of consistent transcription of β-actin among all samples by species, indicating its use as a reference gene. Transcription of IL-1β, TNF-α, and IL-10 in RES pre-infection was measurable in 32/45, 10/45, and 9/45 samples, respectively. For RES post-infection, relative transcription was measurable in 21/22, 7/22, and 7/22 samples, respectively. Transcription of IL-1β, TNF-α, and IL-10 in EBT was measureable in 6/14, 13/14, and 5/14 samples, respectively (Table 5).

4. Discussion

We designed cytokine assays for one reference gene and three immune-mediating cytokine mRNA targets in two species of chelonians following MIQE guidelines [6]. These assays can be used to define a baseline quantitative range for mRNA transcription and for subsequent comparison to populations experiencing viral infections, including ranavirus. It will allow us to elucidate patterns of host-pathogen interaction that lead to different outcomes in morbidity and mortality between individuals and populations. In manatees, this technique has been applied to observe the difference in transcription levels between sites, with higher transcription on the east coast versus those on the west coast of Florida [9]. In that study, the sample size was too small to determine if temperature had an effect, specifically through cold shock syndrome, but it warrants further consideration. The technique can be broadly applied to other species of concern, given the appropriate development of species-specific assays. With the importance of temperature in ectotherm immune response, the transcription of cytokines have the potential to offer insight into the trade-off that occurs within the host at different temperatures within the preferred optimal temperature zone [26]. Understanding the balance can inform management decisions in captive and free-range ectotherm populations experiencing single or multiple stressors that threaten their viability.

In order to achieve this goal, we need to consider the practicality of the methods developed and implemented to run these cytokine assays. The buffy coat isolation technique followed by RNA extraction was successful for consistent yield and quality values. This is likely related to the concentration of white blood cells, which produce the cytokine transcript targets. However, the approach is delayed for up to several hours after sampling, and is ultimately time-consuming. A potential solution is to collect blood and store the sample directly into RNALater, but further work is needed to determine the ability to produce high quality RNA suitable for downstream applications. The synthesis of cDNA is also a potential variable impacting the outcome of the RT-qPCR analysis. We used a mix of oligo-dT primers and random primers to reverse transcribe cDNA from the RNA template. Additionally, we used the two-step RT-qPCR method for sample stability and flexibility with downstream analyses, which has been found comparable to the one-step method [32]. To mitigate the risk of contamination we used RNA-only lab equipment and an additional genomic DNA elimination buffer as an additional precaution.

We were unable to successfully sequence product for IL-10 for either target species using conventional PCR. The inability may have been due to the following reasons: (1) the primer design was inadequate, (2) the selected samples did not contain the sequence, (3) these species do not produce the target cytokines, or (4) the predicted sequences are not close enough to the actual sequences. The predicted values and the successfully sequenced products (IL-1β and TNFα) were 99% homologous, such that that the sequence for IL-10 would likely closely align with predicted sequences in GenBank. Based on our preliminary test results, evidence of IL-10 transcription was available for 21/81 turtle RNA samples. The low transcription levels are similar to those measured in ferrets [7]and manatees [9]. Positive or near-positive samples will be the focus for more conventional PCR tests, to confirm the genetic identity of the target mRNA transcript and to potentially enhance the design of the primer-probes and lower the limit of detection for less common transcripts. Additionally, the timing of sampling may have impacted the results. Our assays can be applied in cell cultures to test more time points than would be possible *in vivo*, which in turn can better guide sampling protocol within experimental design.

With the development of this assay, we aim to enhance our understanding of at-risk chelonian population health. By designing the appropriate tools to define relative transcription levels, we can establish normal and abnormal responses to population stressors in multiple repeated samples from species that only allow a small volume of blood collection. Because of the non-specific nature of inflammation, the

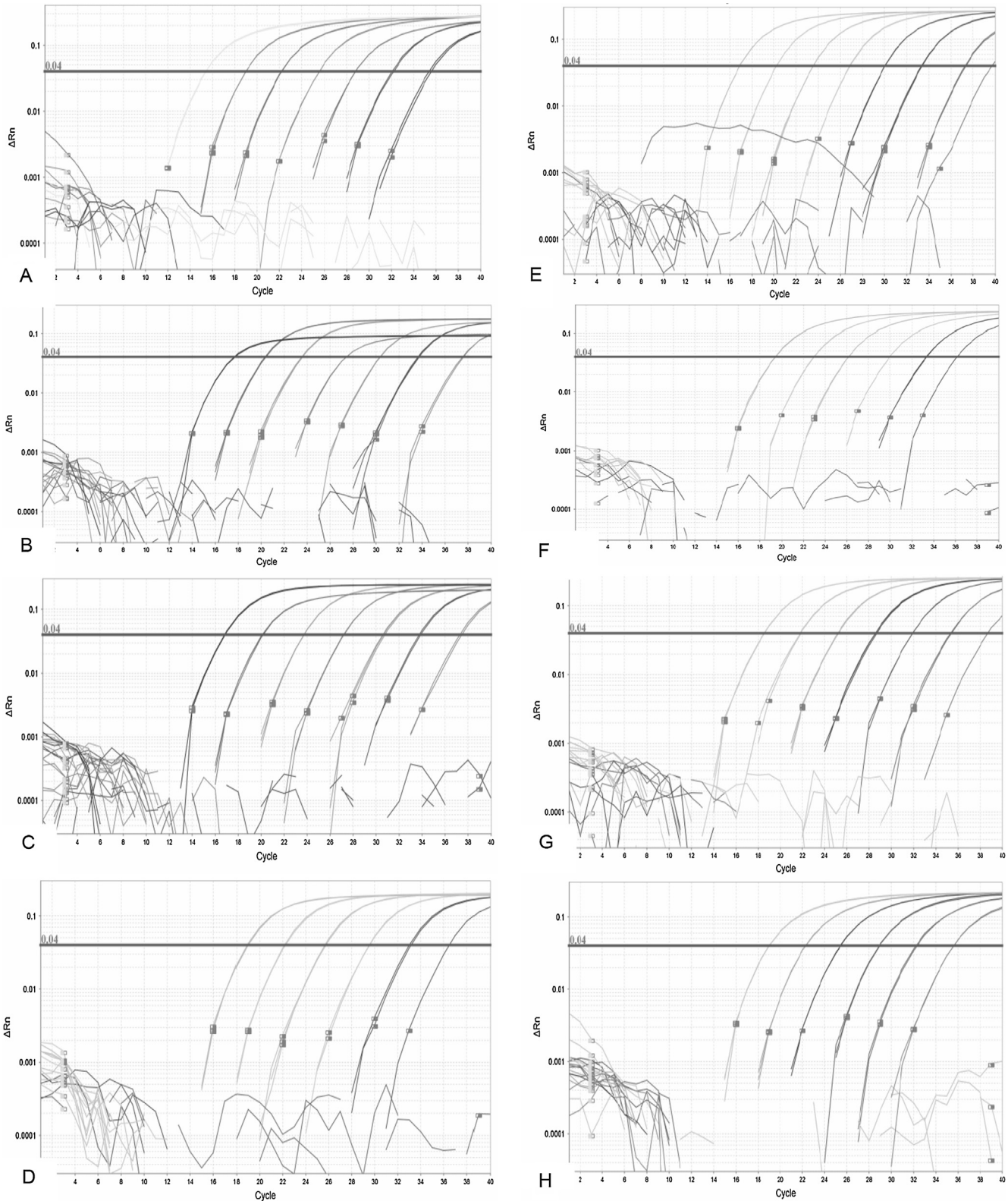

Fig. 1. Amplification plots for validated cytokine mRNA transcription assays for two chelonian species – red-eared sliders (RES, *Trachemys scripta elegans*) and eastern box turtles (EBT, *Terrapene carolina carolina*): (A) RES beta actin 212, (B) RES beta actin 43, (C) EBT conserved beta actin, (D) tumor necrosis factor alpha, (E) interleukin-1 beta 16, (F) interleukin-1beta 55, (G) interleukin-10 80, (H) interleukin-10 316. The number denotes the base pair at which the forward hydrolysis primer begins to differentiate sets. Dilutions ranged from 10^7 to 10^1 copies per reaction, made from linearized synthetic plasmid DNA.

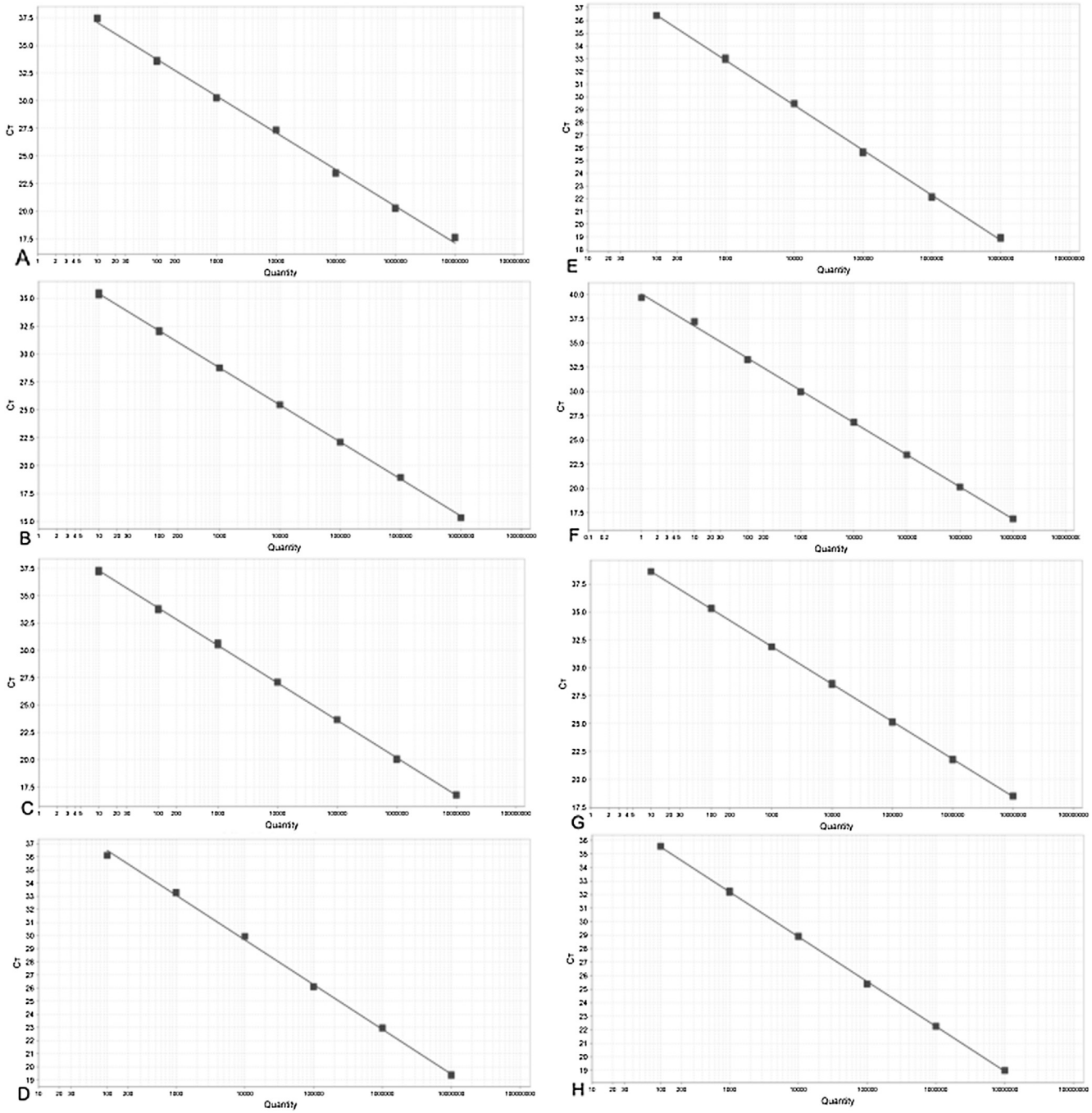

Fig. 2. Standard curves evaluated for validated cytokine mRNA transcription assays for two chelonian species – red-eared sliders (RES, *Trachemys scripta elegans*) and eastern box turtles (EBT, *Terrapene carolina carolina*): (A) RES beta actin 212, (B) RES beta actin 43, (C) EBT conserved beta actin, (D) tumor necrosis factor alpha, (E) interleukin-1 beta 16, (F) interleukin-1beta 55, (G) interleukin-10 80, (H) interleukin-10 316. The number denotes the base pair at which the forward hydrolysis primer begins to differentiate sets. Dilutions ranged from 10^7 to 10^1 copies per reaction, made from linearized synthetic plasmid DNA. All values fall within an acceptable range of efficiency, 85–110%, and an $R^2 > 0.98$.

range of values may include the transcription of primarily inflammatory cytokines, even in apparently healthy animals like our red-eared sliders prior to viral infection. In general, we observed expected pattern of more samples and greater quantity of IL1B transcription in post-infection sliders compared to pre-infection. However, TNF and IL10 were more similar between groups, likely because of their low transcription values. Timing and quantity of transcription can be used to establish host inflammatory response to a specific etiology. Therefore, the use of additional time points will be necessary to better understand host response to viral infection, and to provide robust statistical analyses rather than pattern observations.

As a group, chelonians are appropriate research subjects because of their vulnerable status [25]. Additionally, their immune response as ectotherms, driven by temperature and heavily reliant upon the innate system [26] is the primary target of our novel RT-qPCR assays. By establishing response patterns, we can observe the influence of disease outbreaks and the chelonian host response that plays a role in outcome. Then, when a disruptive event occurs such as an outbreak of disease, rapid environmental change, or any stimuli that might induce an immune response, samples can be compared to baseline relative levels. Ultimately, the host chelonian response can be quantified to evaluate health and potentially predict outcomes based on relative cytokine

Table 4
Intra-assay and inter-assay comparison between critical threshold (CT) values for cytokine mRNA primer- probe sets. Each dilution was 10^7 copies to 1 copy of target sequence, and all were tested in triplicate. (CV = Coefficient of variation). Assays were developed for two chelonian species, red-eared sliders (*Trachemys scripta elegans*) and eastern box turtles (*Terrapene carolina carolina*).

Dilution	Mean -Intra	SD- Intra	CV %- Intra	Mean - Inter	SD - Inter	CV % - Inter
Red-eared slider B actin 212						
10^7	15.33	0.00	0.00	15.33	0.00	0.00
10^6	18.94	0.01	0.03	18.94	0.01	0.03
10^5	22.11	0.03	0.12	22.11	0.03	0.12
10^4	25.47	0.00	0.00	25.47	0.00	0.00
10^3	28.76	0.01	0.03	28.76	0.01	0.03
10^2	32.04	0.09	0.28	32.04	0.09	0.28
10^1	35.40	0.15	0.42	35.40	0.15	0.42
Red-eared slider B actin 43						
10^7	17.63	0.06	0.33	17.63	0.06	0.33
10^6	20.28	0.05	0.22	20.28	0.05	0.22
10^5	23.47	0.05	0.20	23.47	0.05	0.20
10^4	27.35	0.01	0.05	27.35	0.01	0.05
10^3	30.25	0.03	0.11	30.25	0.03	0.11
10^2	33.60	0.06	0.19	33.60	0.06	0.19
10^1	37.45	0.07	0.18	37.45	0.07	0.18
Consensus Red-eared slider and eastern box turtle B actin						
10^7	16.78	0.03	0.17	16.78	0.05	0.28
10^6	20.05	0.04	0.21	20.19	0.16	0.81
10^5	23.65	0.01	0.04	23.60	0.07	0.30
10^4	27.10	0.02	0.08	27.14	0.05	0.20
10^3	30.59	0.10	0.33	30.61	0.09	0.30
10^2	33.76	0.10	0.29	33.78	0.11	0.34
10^1	37.26	0.12	0.32	37.48	0.28	0.75
10^0						
Consensus Red-eared slider and eastern box turtles ILB-16						
10^7	19.20	0.03	0.14	19.28	0.10	0.51
10^6	23.27	0.02	0.08	23.36	0.10	0.41
10^5	26.96	0.01	0.03	27.16	0.28	1.02
10^4	31.24	0.07	0.22	31.36	0.14	0.44
10^3	35.51	0.10	0.27			
Consensus Red-eared slider and eastern box turtles ILB-55						
10^7	16.87	0.01	0.09	16.92	0.04	0.26
10^6	20.16	0.00	0.01	20.17	0.02	0.10
10^5	23.48	0.00	0.01	23.51	0.04	0.17
10^4	26.83	0.03	0.11	26.74	0.08	0.31
10^3	29.96	0.02	0.07	29.96	0.02	0.05
10^2	33.28	0.03	0.08	33.37	0.14	0.42
10^1	37.19	0.04	0.12	36.99	0.23	0.62
Consensus Red-eared slider and eastern box turtles TNF-alpha						
10^7	19.17	0.07	0.38	19.27	0.12	0.65
10^6	22.80	0.09	0.38	22.88	0.11	0.47
10^5	25.91	0.07	0.25	26.00	0.12	0.45
10^4	28.86			29.40	0.76	2.58
10^3	33.20	0.03	0.08	33.24	0.05	0.16
10^2	36.18	0.11	0.29	36.16	0.09	0.24
Consensus Red-eared slider and eastern box turtles IL10-80						
10^7	18.51	0.02	0.11	18.55	0.04	0.21
10^6	21.79	0.05	0.21	21.79	0.05	0.25
10^5	25.14	0.04	0.14	25.12	0.03	0.12
10^4	28.54	0.07	0.25	28.57	0.07	0.23
10^3	31.88	0.01	0.02	31.84	0.08	0.25
10^2	35.31	0.05	0.13	35.31	0.12	0.33
Consensus Red-eared slider and eastern box turtles IL10-316						
10^7	19.02	0.02	0.10	19.00	0.03	0.13
10^6	22.30	0.03	0.12	22.28	0.03	0.15
10^5	25.62	0.03	0.12	25.50	0.15	0.57
10^4	29.07	0.03	0.10	28.97	0.09	0.33
10^3	32.37	0.01	0.03	32.29	0.10	0.32
10^2	35.63	0.01	0.03	35.60	0.03	0.09

Table 5
Transcription levels of the cytokines interleukin 1 beta (IL1B), tumor necrosis factor alpha (TNF), and interleukin (IL10) relative to β-actin expression in a subset of 81 RNA samples tested during RT-qPCR assay validation in experimental red-eared sliders (*Trachemys scripta elegans*, RES, 67 samples [45 pre-infection, 22 post-infection with Frog virus 3]) and free-ranging eastern box turtles (*Terrapene carolina carolina*, EBT, 14 samples).

	EBT	RES (pre-infection)	RES (post-infection)
IL1B Range	0–4.779	0–0.505	0–2.061
IL1B Mean	0.527	0.077	0.510
IL1B SD	1.209	0.118	0.628
TNF Range	0–0.046	0–0.003	0 – 0.020
TNF Mean	0.004	0.0001	0.002
TNF SD	0.012	0.0005	0.005
IL10 Range	0–0.074	0 – 0.058	0 – 0.012
IL10 Mean	0.006	0.002	0.002
IL10 SD	0.019	0.009	0.003

transcription as a significant parameter.

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