A Real-time Quantitative Polymerase Chain Reaction for the Specific Detection of *Hammondia Hammondi* and Its Differentiation from *Toxoplasma Gondii*

Gereon Schares (Gereon.Schares@fli.de)
Friedrich-Loeffler-Institut https://orcid.org/0000-0002-3217-289X
Majda Globokar Vrhovec
IDEXX Laboratories Inc
Mareen Tuschy
Friedrich-Loeffler-Institute: Friedrich-Loeffler-Institut Bundesforschungsinstitut fur Tiergesundheit
Maike Joeres
Friedrich-Loeffler-Institute: Friedrich-Loeffler-Institut Bundesforschungsinstitut fur Tiergesundheit
Andrea Bärwald
Friedrich-Loeffler-Institute: Friedrich-Loeffler-Institut Bundesforschungsinstitut fur Tiergesundheit
Bretislav Koudela
VFU FVHE: Veterinarni a farmaceuticka univerzita Brno Fakulta veterinarni hygieny a ekologie
Jitender P. Dubey
USDA-ARS BARC: USDA-ARS Beltsville Agricultural Research Center
Pavlo Maksimov
Friedrich-Loeffler-Institute: Friedrich-Loeffler-Institut Bundesforschungsinstitut fur Tiergesundheit
Franz J. Conraths
Friedrich-Loeffler-Institute: Friedrich-Loeffler-Institut Bundesforschungsinstitut fur Tiergesundheit

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Abstract

Introduction: *Hammondia hammondi* and *Toxoplasma gondii* are closely related protozoan parasites, but only *T. gondii* is zoonotic . Both species use felids as definitive hosts and cannot be differentiated by oocyst morphology. In *T. gondii*, a 529 bp repetitive element (TgREP-529) is of utmost diagnostic importance for PCR diagnostic tests. We identified a similar repetitive region in the *H. hammondi* genome (HhamREP-529).

Material and Methods: Based on reported sequences, primers and probes were selected in silico and optimal primer probe combinations were explored, also by including previously published primers. The analytical sensitivity was tested using serial dilutions of oocyst DNA. For testing analytical specificity, DNA isolated from several related species were used as controls. The newly established TaqMan PCR (Hham-qPCR1) was applied to tissues collected from *H. hammondi*-infected gamma-interferon knockout (GKO) mice at varying time points post infection.

Results: Ten forward and six reverse primers were tested in varying combinations. Four potentially suitable dual-labelled probes were selected. One set based on the primer pair (Hham275F, Hham81R) and the probe (Hham222P) yielded optimal results. In addition to excellent analytic specificity, the assay revealed an analytical sensitivity of genome equivalents of less than 1 oocyst. Investigation of the tissue distribution in GKO mice revealed the presence of parasite DNA in all examined organs, but to a varying extent suggesting 100- to 10.000-fold differences in parasitic loads between tissues in the chronic state of infection, 42 days post infection.

Discussion: The use of the 529 bp repeat of *H. hammondi* is suitable for establishing a quantitative real-time PCR assay because this repeat probably exists about 200-times in the genome of a single organism, like its counterpart in *T. gondii*. Although there was enough sequence data available, only few of the primers predicted in silico revealed sufficient amplification; the identification of a suitable probe was also difficult. This is in accord with our previous observations on considerable variability in the 529 bp repetitive element of *H. hammondi*.

Conclusions: The *H. hammondi* real-time PCR represents an important novel diagnostic tool for epidemiological and cell-biological studies on this and related parasites.

1. Introduction

Hammondia hammondi is a coccidian parasite, which is closely related to the zoonotic protozoan *Toxoplasma gondii* [1, 2]. Similar to *T. gondii*, *H. hammondi* uses felids such as the domestic cat as definitive hosts [3]. Oocysts of *T. gondii* and *H. hammondi* are morphologically indistinguishable [3, 4]. Thus, faecal examinations solely based on microscopy cannot be used to estimate the prevalence of *T. gondii* or *H. hammondi* oocysts in feline hosts [5, 6, 7].

Laboratory mice, rats, hamsters, guinea pigs, wild rodents, rabbits, goats and dogs are susceptible to infection with *H. hammondi* oocysts (Eydelloth, M., 1977. Experimentelle Untersuchungen über das Wirtspektrum von *Hammondia hammondi*. Dissertation, Veterinary Faculty Munich, Germany; [3, 6, 8, 9, 10, 11, 12, 13]. While monkeys [14] are also experimental intermediate hosts of *H. hammondi*, no *H. hammondi* infection has so far been demonstrated in humans. No avian intermediate hosts, including chickens [15], quails [12] and pigeons [9, 12] could be infected with *H. hammondi*, which suggests avians are not its intermediate hosts [7].

While several PCRs are available for the diagnosis of *T. gondii* infections, only a few diagnostic PCRs for *H. hammondi* have been reported so far. Those for *T. gondii* are based on a variety of targets, including the B1 gene [16], the ITS-1 region of the ribosomal DNA [17] and a 529-repetitive element [18, 19]. The ones reported for *H. hammondi* are end-point PCRs, which use as targets the ITS-1 rDNA [20] or a 529 bp repeat, which is similar to the *T. gondii* repetitive element (TgREP-529) [6]. To the our knowledge, no real-time PCR to diagnose *H. hammondi* infections has so far been published. The availability of a real-time PCR will allow simplifying *H. hammondi* prevalence estimates. It will also allow the detection and quantification of oocysts of this parasite in feline faeces and in tissues of natural and experimental intermediate hosts.

Since the 529 bp repetitive element (TgREP-529) exists up to about 200-times in the genome of a single *T. gondii* organism as recently shown by third-generation sequencing [21] and sequence information on a homologue of this repetitive element in *H. hammondi* was readily available from prior studies [6], we decided to establish a REP529-based real time PCR for *H. hammondi*. Unexpectedly, we faced several problems, when we tried to establish a sensitive primer and probe combination, probably due to sequence variations between individual repeats as observed earlier [6].

2. Material And Methods

2.1. Parasite strains to generate reference DNAs

Field isolates of *H. hammondi* oocyst were made available by IDEXX Laboratories, Ludwigsburg, Germany, and the University of Veterinary and Pharmaceutical Sciences, Brno, Czech Republic, in the frame of projects that aimed at obtaining European *T. gondii* isolates (Supplementary data Table S1). Faecal samples were tested by flotation and examined microscopically as described previously [7]. If *T. gondii*-like oocysts were detected, purified oocysts were genotyped at the species level by PCR using DNA extracted from oocysts. Occasionally, gamma interferon gene knock out (GKO) mice (C.129S7 (B6)-Ifngtm1Ts/J, The Jackson Laboratory, Bar Harbor, Maine, USA) were in addition inoculated with sporulated oocysts via oral gavage, parasites recovered as described [22] and genotyped. One *H. hammondi* oocyst isolate from Iran had been kindly provided by Dr. Morteza Hosseininejad, Faculty of Veterinary Medicine, Shahr-e Kord, Iran, in 2010. Oocysts of the isolate H.H.34 were from United States, for which whole genome sequences were recently obtained (GenBank: AHJH01004981.1). H.H.34 oocysts

were used to infect GKO mice to obtain tissue stages for DNA extraction and DNA was also extracted from the oocysts (Supplementary data Table S1, S2).

To confirm the specificity of the Hham-qPCR1, we used DNA from three *T. gondii* strains (RH, ME49, NED), *N. caninum* (Nc-1), *Besnoitia besnoiti* (Evora), *Hammondia heydorni*, *Eimeria bovis*, *Cystoisospora felis*, *C. rivolta*, *C. burrowsi*, *C. canis*, *Cryptosporidium parvum*, *Sarcocystis hirsuta*, *S. bovifelis*. *S. hominis*, *S. cruzi*, *Giardia* spp. and *Tritrichomonas foetus* in addition to DNA isolated from *H. hammondi*. *Toxoplasma gondii*, *N. caninum* and *B. besnoiti* were obtained by in-vitro cultivation of the parasites, which yielded reference samples consisting of 100 ng/µl concentrated pure DNA. For the remaining oocyst reference DNA samples, DNA was extracted from field isolates (*Hammondia heydorni*, *Cystoisospora* spp., *Sarcocystis* spp.; 10^4 – 10^5 oocysts or sporocysts) yielding 100 µl DNA for each parasite. *Eimeria bovis* oocysts were kindly provided by Dr. Christian Bauer, Institute for Parasitology, Giessen, Germany. Oocysts of *Cryptosporidium parvum* (Germany) were kindly provided by Prof. Dr. A. Daugschies, Institute of Parasitology Leipzig, Germany. *Giardia* spp. and *Tritrichomonas foetus* DNA were kindly provided by Dr. Christian Klotz, Robert Koch-Institut, Berlin, Germany and Dr. Klaus Henning, Friedrich-Loeffler-Institut, Jena, Germany.

2.2. Experimental infections of mice

GKO mice (C.129S7(B6)-Ifngtm1Ts/J, The Jackson Laboratory, Bar Harbor, Maine, USA) mice (n = 28) were orally inoculated with different isolates and varying doses depending on the availability of field isolates in experiments to isolate *T. gondii* or to trigger the permanent growth of *H. hammondi* in mice and eventually in cell-culture (Supplementary Table S2). Sporulated oocysts were counted using a Neubauer chamber and mice infected via oral gavage as described [22].

2.3. DNA extraction

Oocysts were isolated from faeces using combined sedimentation and flotation by adding 13 ml concentrated sucrose (specific gravity of 1.3) to 1 ml faecal sediment as described previously [23]. Floating oocysts were collected, washed three times by centrifugation (1100 x g, 7 min, without brake) and a 5- to 10-fold volume of PBS. DNA was extracted from the final pellet using a Phenol/Chloroform purification method previously described [22] or the NucleoSpin Soil kit (Macherey and Nagel, Düren, Germany) following the manufacturer's recommendations.

2.4. Conventional endpoint PCRs

To confirm the presence of *H. hammondi* in oocyst samples, a previously published end-point PCR was applied as described to examine oocyst DNA [4].

To test DNA extracted from oocyst samples for coccidian DNA, a PCR was performed using the common apicomplexan small subunit ribosomal DNA (SSU-rDNA) primers COC-1 and COC-2 [24]. Primers were used at a final concentration of 0.5 mM and dNTPs at a final concentration of 250 mM each (Stratec Molecular GmbH, Berlin, Germany). Taq polymerase (Stratec Molecular GmbH, Berlin, Germany) was used at 1U/25 µl with the buffer system supplied with the enzyme. The PCR cycling conditions were the same

as previously reported [25]. To confirm the presence of *Sarcocystis* spp., primers SarcoFint and SarcoRint specific for *Sarcocystis* spp. SSU-rDNA were used as previously described [26]. To test for *Giardia* spp. DNA, the published primers AS1 GiardiaF and AS2 GiradiaR [27] were used and for *T. foetus* DNA, we used the primer pair TFR1/TFR2 [28]. All reagents, except for primers, were the same as described above. They were used in the same concentrations as described for the coccidian PCR. The PCR cycling conditions were 94 °C for 5 min, followed by 35 cycles of 60 °C for 1 min, 72 °C for 1 min and 94 °C for 1 min. The PCR ended with a final extension at 72 °C for 10 min.

2.5. Identification of primer pairs suitable for amplifying *H. hammondi* DNA

To identify optimal primer pairs, a SybrGreen real-time PCR was performed using DNA (D9494) from a *H. hammondi* isolate (VB919008), diluted to resemble the DNA of approximately 5 oocysts per µl DNA. All possible combinations of forward and reverse primers (Supplementary Table S3) were checked in a SybrGreen real-time PCR using the iTaq™ Universal SYBR® Green Super mix (Biorad Laboratories GmbH, Munich, Germany) to test the *H. hammondi* template in comparison to a no-template control (DNA-grade water). Each primer pair was evaluated twice in two independent SybrGreen real-time PCRs and the results were recorded as mean $\Delta Ct_{pos-neg}$, i.e. the ΔCt between results (Ct values) for the *H. hammondi* template and the no-template control (Supplementary Table S4).

2.6. Novel real-time PCR to detect *H. hammondi* DNA

The quantitative TaqMan real-time PCR was performed as described previously [25], including the integration of an Internal Control (IC) system [25, 29]. The novel real-time PCR employing the optimal primer-probe combination was designated Hham-qPCR1. To monitor inhibition in real-time PCR, a heterologous plasmid DNA resembling the enhanced green fluorescent protein (EGFP) gene [29] was added to the reaction mix including the primers EGFP1-F, EGFP1-R and the probe EGFP1 [25]. A 712 bp fragment of the EGFP gene was amplified as the EGFP template and cloned into the pGEMTeasy standard cloning vector (Promega, Walldorf, Germany) in reverse orientation to obtain the IC-2 DNA (pGEM-EGFP2-rev). The amount of the IC-2 DNA added to each reaction was adjusted in such a way that it resulted in a Cq value of about 32 in the real-time PCR. Reactions were performed in a final volume of 20 µl using a commercial master-mix (PerfeCTa MultiPlex qPCR ToughMix, Quantabio, VWR International, Darmstadt, Germany) and a CFX96 instrument (Biorad Laboratories GmbH, Munich, Germany). Primers and probes (Supplementary Table S3) were purchased from MWG-Biotech (Ebersberg, Germany).

Standard concentrations for primers (500 nM, Supplementary Table S3) and probes (100 nM for *H. hammondi*-probes 160 nM, Cy5 labelled, Supplementary Table S3; EGFP1, HEX labelled, G Schares, JP Dubey, B Rosenthal, M Tuschy, A Bärwald and FJ Conraths [25]) were applied. These standard concentrations were also applied to the finally selected primer-probe combination (Hham275F, Hham81R, Hham222P). The cycling conditions in the Hham-qPCR1 real-time PCR were 95.0 °C (5 min, initial denaturation), followed by 45 cycles, during which the samples were first incubated at 95.0 °C for 10 s and then at 60.0 °C for 30 s. After each cycle, light emission by the fluorophore was measured. Real-time

PCR results were analysed using the CFX manager software Version 1.6 (Biorad Laboratories GmbH, Munich, Germany).

2.7. Sensitivity and specificity of Hham-qPCR1

The analytical sensitivity was determined using a *H. hammondi* oocysts isolate with a high sporulation rate (P18/2900; sporulation rate 84%). Ten-fold dilutions of three DNA samples isolated from 10^5 oocysts were tested by the Hham-qPCR1 in two-fold repetition including the EGFP internal control.

To confirm that the novel PCR recognized a broad range of *H. hammondi* specimens, oocyst isolates from Germany and other countries were tested by Hham-qPCR1. These DNA samples included oocysts DNA isolated from 22 *H. hammondi* samples of different origin from Germany, 4 samples from Denmark, 2 from Austria, each one from France, the Czech Republic and the USA, i.e. n = 28 additional samples in total (Supplementary Table S1). The presence of *H. hammondi* had been confirmed by our previously published end-point PCR [6]. All these samples had tested negative in a Tg-qPCR [30, 31].

The analytical specificity was confirmed using DNA samples of parasites closely related to *H. hammondi*, which were mentioned in Sect. 2.1. The presence of DNA in these samples was confirmed by SSU-rDNA end-point PCR as detailed in Sect. 2.4.

2.8. Statistical analysis

Calculations of median and standard deviations were performed using EXCEL 2019, Microsoft Office (Microsoft Corporation, Seattle, WA, USA).

To determine the relatedness of real time PCR results and oocyst concentration in samples, linear regression was performed using the “lm” command in R, version 3.5.3 (R Core Team, 2017). For the analysis, oocyst concentration per ml were log10-transformed.

Figures were assembled using R, versions 3.5.3 or 4.0.0 (packages “ggplot2”, “reshape” and “scales”). Box plots displaying real time PCR data of infected mice show the median (line), 25th and 75th percentiles (box), the 1.5-fold interquartile ranges (whiskers) and outliers (dots); negative PCR results were counted with a Ct value of 45.

3. Results

3.1. Location of primers

Potentially suitable primers and probes (Supplementary Table S3) for two 5’-nuclease quantitative real time PCR assays were selected by in-silico methods (Fig. 2). To this end, published sequences (n = 9) of the 529 bp repeat of *H. hammondi* were downloaded from GenBank™ (KC223619, JX477424, EU493279, EU493280, EU493281, EU493282,EU493283, EU493284, EU493285) and aligned with Geneious 10.0.9 (Multiple Geneious Alignment, Global alignment with free end gaps, Gap open penalty 12, Gap extension

penalty 3, refinement iterations 2). Primers were selected to amplify a fragment with a maximum size of about 150–160 bp.

3.2. Identification of primer pairs optimal to amplify *H. hammondi* DNA

In total, 39 primer pairs were evaluated with a SybrGreen assay and *H. hammondi* reference DNA D9494. Out of 51 primer pairs tested, n = 22 revealed $\Delta Ct_{pos-neg}$ values of > 4. Five of these 22 pairs revealed a large difference between two independent tests and in 17, the coefficient of variation of $\Delta Ct_{pos-neg}$ (Supplementary Table S4) was less than 0.4. These 17 primer pairs were further validated with one up to four of the probes Hham55P, Hham75P, Hham110P or Hham222P (Supplementary Table S3) depending on the location of primers and probes (Fig. 1). Eleven of the 28 primer-probe combinations were excluded due to cross-reaction with *T. gondii* DNA (1000 ng). Four of the remaining primer-probe combinations showed mean RFU values > 1000 and mean Ct values of 33.6 to 35.3 in 3–5 independent tests. The finally selected primer-probe combination consisted of the primers Hham275F and Hham81R and the probe Hham222P (Fig. 1; Supplementary Table S3). The analytical sensitivity and specificity were assessed for this primer-probe combination in a multiplex real time PCR, in which EGFP-specific reagents were included as an internal control to assess PCR inhibition, in addition to *H. hammondi*-specific reagents. This assay was designated as the Hham-qPCR1.

3.3. Analytical sensitivity and efficiency of the Hham-qPCR1

The analytical sensitivity of the Hham-qPCR1 was determined using a German *H. hammondi* oocyst isolate, which showed a high sporulation rate. Ten-fold dilutions of three DNA samples isolated separately from 10^5 oocysts each were twice examined by the Hham-qPCR including the EGFP internal control. A PCR efficiency of E = 103.9%, a coefficient of determination of R^2 = 99.7% and a slope of the regression line of 3.231 were recorded (Fig. 3).

To confirm that the Hham-qPCR1 recognizes a broader range of *H. hammondi* isolates, we analysed DNA of oocysts collected from cat faeces. These DNA samples included 18 additional samples from Germany, 4 from Denmark, 2 from Austria and 1 from France, the Czech Republic and the USA each, i.e. a total of 28 samples. In the new Hham-qPCR1 these samples yielded Ct values between 15.9 and 28.3, which is equivalent to the DNA of 10^2-10^3 oocysts per µl. There was a linear relationship between the logarithm (log10) of the number of isolated oocysts per ml and the observed Ct values, which was characterized by coefficient of determination (R^2) of 17.5% (Supplementary data Table S1).

3.4. Analytical specificity

The analytical specificity of Hham-qPCR1 was assessed using DNA samples of parasites closely related to *H. hammondi*, i.e. *T. gondii* (strains RH, ME49, NED), *N. caninum* (Nc-1), *B. besnoiti* (Evora), *H. heydorni*, *Eimeria bovis*, *Isospora felis*, *I. rivolta*, *I. burrowsi*, *I. canis*, *Cryptosporidium parvum*, *Sarcocystis hirsuta*, *S. bovifelis*. *S. hominis*, *S. cruzi* and DNA of parasites frequently observed in cat faeces like *Giardia* spp. and

Tritrichomonas foetus. Presence of DNA in these samples was confirmed by end-point PCR (Fig. 4). In the Hham-qPCR1, no amplification with any of these DNA samples was observed.

3.5. Examination of tissue stages of *H. hammondi*

Tissues of 28 GKO mice infected with *H. hammondi* for varying time periods were therefore available, from which DNA was extracted and tested by Hham-qPCR1 (Fig. 5a, b). From each of 28 mice, seven tissues (brain, heart, lung, liver, kidney, spleen and the proximal hind- limb musculature) had been collected. In the first days post infection (DPI 3, DPI 6), the median Ct values in Hham-qPCR1 ranged between 22.3 and 31.3 for organs including mostly muscle tissue (except for the distal hind-limb and tongue, 39.2 or 38.9, respectively); in contrast, median Ct values in brain ranged only from 36.5 to 39.9 (Fig. 5a). On DPI 42, skeletal muscle (i.e. proximal hind-limb musculature) showed the lowest median Ct values (14.2) of all tissues in this comparison followed by heart (17.3) and lung (20.4). Of the remaining organs, brain and spleen showed similar median Ct values (24.9 and 25.4). Liver and kidney also yielded Ct values in the same range (27.4 and 29.4). Later during infection (DPI 74–186 and 272), heart and skeletal muscle had the lowest median Ct values followed by lung, brain, kidney and spleen. However, Ct values at DPI 272 were considerably lower than during DPI 74–186 mice (Fig. 5a).

Moreover, muscle tissues obtained from different locations on DPI 3, 6 and 42 were comparatively examined (Fig. 5b). On DPI 3 and 6, heart showed the lowest Ct values of all muscle tissues (median Ct 27.3 and 23.8), while heart and tongue had the highest Ct values on DPI 42 (Fig. 5b). The remaining muscle tissues showed very similar Ct values, i.e. medians ranged from 14.7 to 13.0. In all skeletal muscle tissues, the Ct values had dropped considerably between DPI 6 (Ct 27.0–25.3) and DPI 42 (Ct 10.2–13.3). In brain tissue, a similar decrease in Ct values was observed between DPI 6 and DPI 42. In contrast, for heart, only a ΔCt of 6.5 was observed between the median Ct values of DPI 6 and DPI 42 (Supplementary data Table S5).

4. Discussion

The present study reports on the development and characterization of a 5'-hydrolysis real time PCR (TaqMan PCR) for the quantitative detection of *H. hammondi*. To the best of our knowledge, this seems to be the first real-time PCR for the detection of *H. hammondi* DNA in feline faecal samples or intermediate host tissues.

There is a large number end-point and real-time PCRs for *T. gondii* that target an up to about 200-fold repeated element in the genome of the parasite (TgREP-529). We had identified a similar 529 bp repeat in *H. hammondi* and established the Hham34F/Hham3R end-point PCR to diagnose *H. hammondi* infection several years ago [6]. This PCR was subsequently applied to test feline faecal samples [5, 7], rodent tissues [32] and used in cell-biological studies to elucidate life-cycle difference between *T. gondii* and *H. hammondi* [33]. Although the use of the 529 bp repetitive element of *H. hammondi* seemed to be ideal for establishing a quantitative real-time PCR and enough sequence data were available, no real time PCR had so far been established for *H. hammondi*.

We found that only a small set of the primers, we had predicted in-silico, revealed enough specificity and sensitivity for amplifying *H. hammondi* DNA. The identification of a suitable probe was also more difficult than expected. In a previous study, we had cloned and sequenced several PCR amplification products of the HhamREP-529 and observed considerable sequence variation [6]. It seems possible that these sequence polymorphisms prevented many of the in-vitro selected primers and probes from enough binding.

The HhamREP-529 and the TgREP-529 sequences are similar, but not identical (identity ranging from 93.7 to 97.5%; [6]) and the finally selected *H. hammondi* primers and probes showed a considerable number of miss-matches with respect to the *T. gondii* sequence. To confirm the analytical specificity relative to *T. gondii*, we used DNA samples from the three major European and North American clonal lineages of *T. gondii*. In addition, we confirmed the specificity with a variety of DNA samples from parasite species or genera related to *H. hammondi* or *T. gondii* including parasitic protozoa frequently observed in cat faeces like *Giardia* spp. or *T. foetus*. Our results indicated that the Hham-qPCR1 has an excellent analytical specificity.

When we tested DNA samples of more than 20 *H. hammondi* isolates from Austria, Denmark, France, Germany and the USA (oocysts), the Czech Republic (oocysts, mouse tissue) and Iran (intermediate host tissue), we found no indication that our Hham-qPCR1 lacks sensitivity for particular isolates. Due to the limited knowledge on the global population structure of *H. hammondi*, we cannot exclude, however, that yet unnoticed lineages of *H. hammondi* exist, which may be genetically diverse. If there are further *H. hammondi* lineages, the possibility remains that our primer-probe combination could show limited or no binding to the DNA of such lineages. However, the experience with *T. gondii*, where there are several genetically diverse haplogroups, may suggest that TgREP-529 is relatively conserved. In general, only a few failures of TgREP-525-based PCRs for the detection of *T. gondii* have been reported so far [34, 35, 36, 37, 38, 39]. However, there are some cases, in which other PCR assays either based on ITS-1 rDNA [40] or on B1 as a target [41] detected a considerable number of additional *T. gondii* positive DNA samples compared to the number of positive results obtained by TgREP-525- based PCRs.

An earlier study had observed only a few differences among various strains of the canonical clonal lineages regarding the number of TgREP-529 repeats units per organism [42] and a very recent study made similar observation [21]. It has been discussed, but so far not investigated, to which extent sequence polymorphisms between the tested canonical clonal lineages contributed to these findings [42]. It must be assessed in future studies, if there are differences regarding the numbers of HhamREP-529 repeats in strains of *H. hammondi*.

We validated the analytical sensitivity of the Hham-qPCR1 with oocyst DNA. Similar to our previous results with an end-point PCR [6], the analytical sensitivity of the Hham-qPCR1 was equivalent to the DNA content of 0.1 oocysts. Considering that 200 µg faeces are usually used and yield 100 µl DNA solution, the limit of detection comes close to 5 oocysts per g faeces, which is comparable to the analytical sensitivity reported for a *T. gondii* copro-PCR [43, 44] and a *Besnoitia darlingi* real time PCR [25]. For

comparison, the microscopical evaluation of faecal flotation by sucrose density centrifugation showed a limit of detection of 250 oocysts per g faeces [45].

To demonstrate the practical value of the novel Hham-qPCR1 in examining infections of intermediate hosts, we used tissue samples of GKO mice inoculated with *H. hammondi* oocysts. In contrast to infection with *T. gondii*, *H. hammondi* infections generally become chronic in GKO mice without causing disease (own unpublished data). If clinical signs were mentioned, they were not described in detail [3, 5]. There are indications, however, that high doses of 10^6 oocysts cause serious illness in < 10% of inoculated GKO mice [5]. The reason why *H. hammondi* is not as virulent seems to be that *H. hammondi* replicates only a few times during the tachyzoite stage and eventually differentiates into bradyzoites [33] that does no longer cause cell death or other harm to the host. As evidenced by laborious and time consuming histological examinations, cyst-producing (i.e. BAG-1 positive) stages were seen as early as 7 days post inoculation in mice, suggesting that at least until then, *H. hammondi* had multiplied in the tachyzoite stage [3]. Tachyzoite-subculture and tissue cyst-in vitro experiments suggest that there is significant tachyzoite multiplication in *H. hammondi* until about 15 to 18 DPI [33]. Thereafter, BAG-1 positive tissue cysts were microscopically visible in heart and skeletal muscles and until 22 DPI also in mesenteric lymph nodes, liver, lung, kidney, brain. After that time, tissue cysts were microscopically only observed in the lung, heart and skeletal muscles [3]. Our real time PCR results corroborate these findings. Chronically infected mice (i.e. mice after 6 weeks of infection), had the lowest Ct values in skeletal muscle, heart and lung tissue (often Ct < 25, and especially in skeletal muscle Ct < 15), but infections were observed frequently also in the remaining organs, but high Ct values dominated (often Ct < 30), if these organs were positive at all. This shows that *H. hammondi* can persist also in other organs than lung, heart or skeletal muscle. The dramatically decreasing Ct values in various skeletal muscles, characterized by ΔCt values of 10.2 to 13.3 between DPI 6 and DPI 42 are remarkable. They suggest a 10^3-fold to 10^4-fold increase in parasite loads in these tissues. In the case of heart tissue, only a ΔCt of 6.5 was observed between DPI 6 and DPI 42, i.e. a 10^2-fold increase in parasite load. This finding may indicate that striated muscles support multiplication and persistence of *H. hammondi* better than smooth muscles. This is in contrast to *T. gondii*, where heart was identified as a predilection site for chronic *T. gondii* infections in many animal species [46]. As *H. hammondi* and *T. gondii* are partially using the same animal species as intermediate host, differences in the host tissue tropism may prevent to some extent interspecies competition in these very closely related parasites.

An increase in the *H. hammondi* parasitic load was also observed in brain, which was characterized by a ΔCt of 11.7, i.e. a 10^4-fold increase in parasite load, between DPI 6 and DPI 42. However, the parasite load was 10^3-times lower than in skeletal muscle, which may suggest that *H. hammondi* tachyzoites reach the brain only late during infection. The increase in the parasite load may be mainly due to a limited tachyzoite and subsequent bradyzoite multiplication in immunologically isolated tissue cysts. This is in accord with earlier observations that there is no cerebral disease in *H. hammondi* inoculated mice [3].

In mice sacrificed after 42–271 DPI, the results suggested that the parasitic load had decreased in all tissues gradually over time. This finding is new, as in the only long-term infection published so far, a single GKO mouse was used for each date from 22 until 127 DPI, in which tissue cysts were observed in lung heart and skeletal muscle until DPI127, but not enumerated [3].

Due to the similar, almost identical morphology of tissue cysts of *T. gondii* and *H. hammondi*, chronic *H. hammondi* infections may represent an interesting model to study the intra-cystic activity of chemical compounds that might be suitable to treat chronic toxoplasmosis. As the definitive discrimination of *H. hammondi* is only possible in the encysted bradyzoite stage and as re-activation of cysts does not seem to occur in this parasite, *H. hammondi* might be used as a model that could provide a readout for the true effect of compounds on encysted *H. hammondi* as a proxy for encysted *T. gondii*. The Hham-qPCR1 described here may thus help to quantify the effect of treatment.

Conclusions

The newly established Hham-qPCR1 assay represents a novel diagnostic tool for epidemiological and cell-biological studies on *H. hammondi* in cats as well in intermediate hosts.

Abbreviations

Tg: *Toxoplasma gondii*

Hham: *Hammondia hammondi*

TgREP-529: 529 bp repetitive element in *Toxoplasma gondii*

HhamREP-529: 529 bp repetitive element in *Hammondia hammondi*

qPCR: real time PCR

Ct: cycle of transition in real time PCR

GKO: IFN-γ knockout mice

Declarations

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Consent for publication

Not applicable.

Availability of data and material

Data supporting the conclusions of this article are included within the article and its additional files. The raw datasets used and analyzed during the present study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

GS designed the study. MG, MJ, PM and GS collected the data. MG, MJ, MT, BK, JPD and AB performed experiments and analyzed the samples. GS statistically analyzed the data. FS, PM and GS interpreted the data. GS, MJ and FJC made major contributions to the writing of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments (bioassays) reported in this publication were approved by the Landesamt für Umwelt Gesundheit und Verbraucherschutz of the German Federal State of Brandenburg (permission 23-2347-8-12-2008) or the Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei of the German Federal State of Mecklenburg- Vorpommern (permission 7221.3-2-023/17).

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Figures

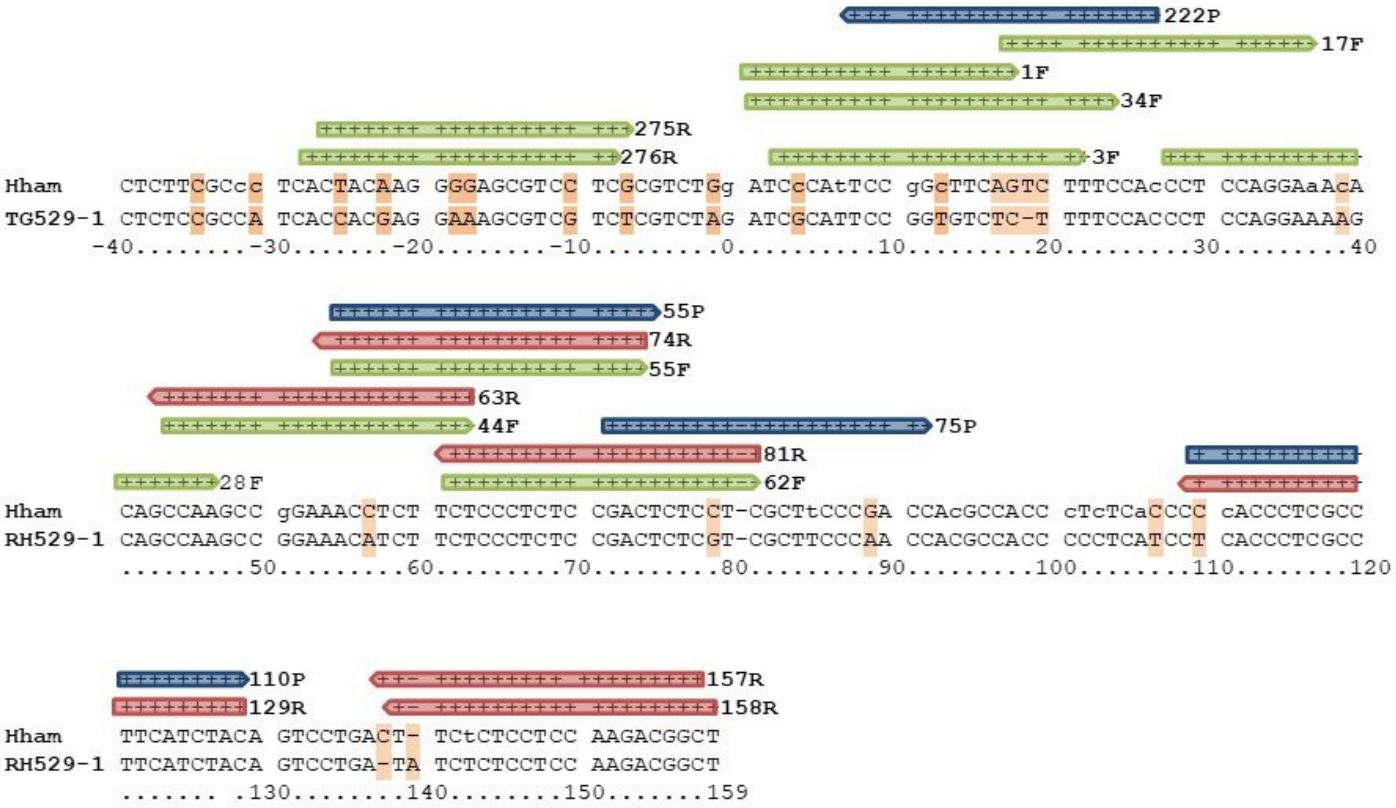

Figure 1

Locations of primers and probes on a fragment of the 529 bp repeat sequence of H. hammondi and T. gondii. Hham, consensus of sequences deposited in Genbank (KC223619, JX477424, EU493279, EU493280, EU493281, EU493282, EU493283, EU493284, EU493285); RH529-1, sequence published by [18]. Further details are provided in the Supplementary Table S3. Colour code: Blue - Probe, Green - Forward primer, Red – Reverse primer; 5‘-end is indicated by arrow-headed ends. Sequence differences are indicated by orange colour. There are several sequence variants of H. hammondi; lower case letters indicate polymorphic sites.

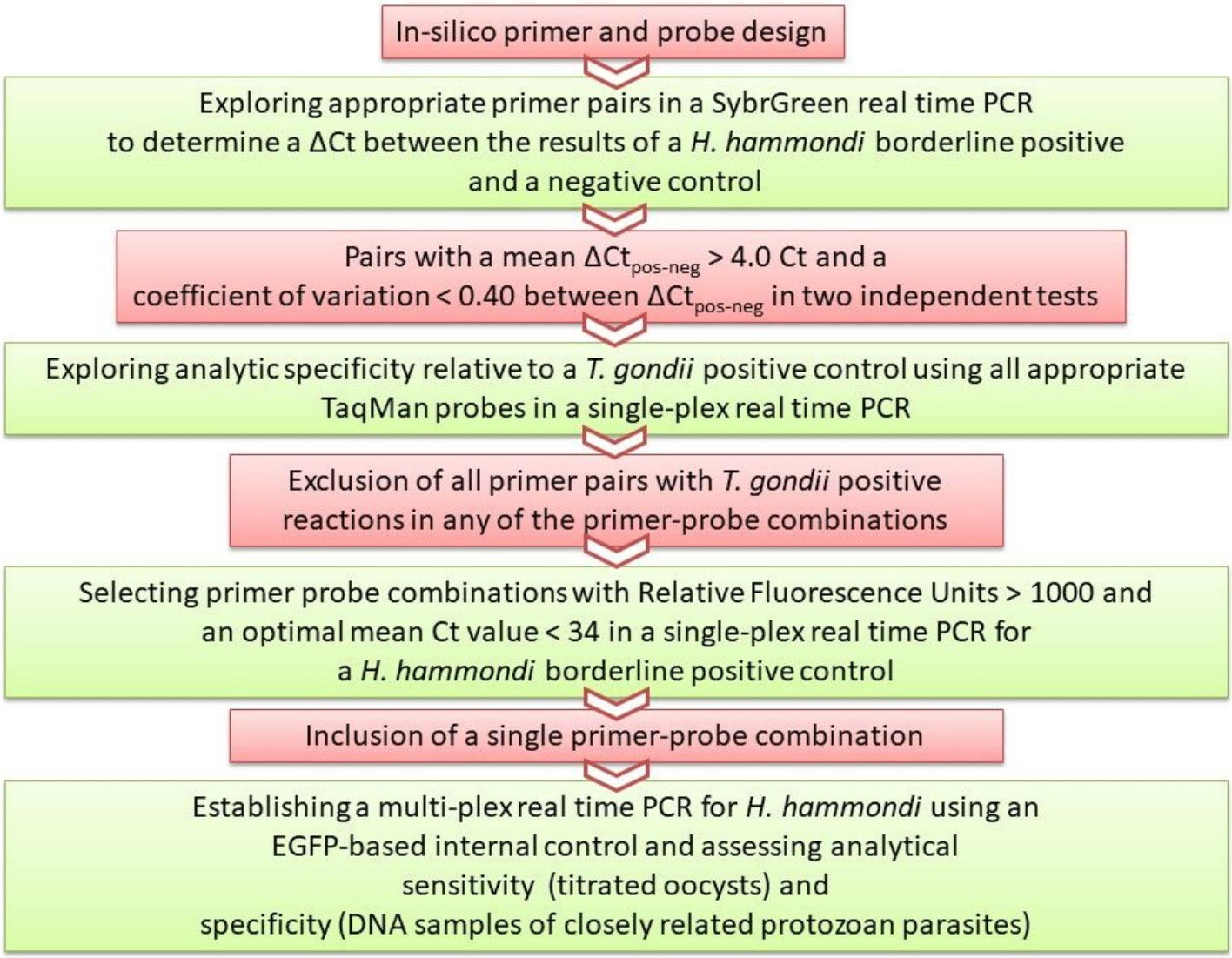

Figure 2

Workflow for establishing the Hham-qPCR1 for diagnosing Hammondia hammondi in oocysts and intermediate host tissues using a sensitive and specific primer-probe combination and an EGFP-based internal control.

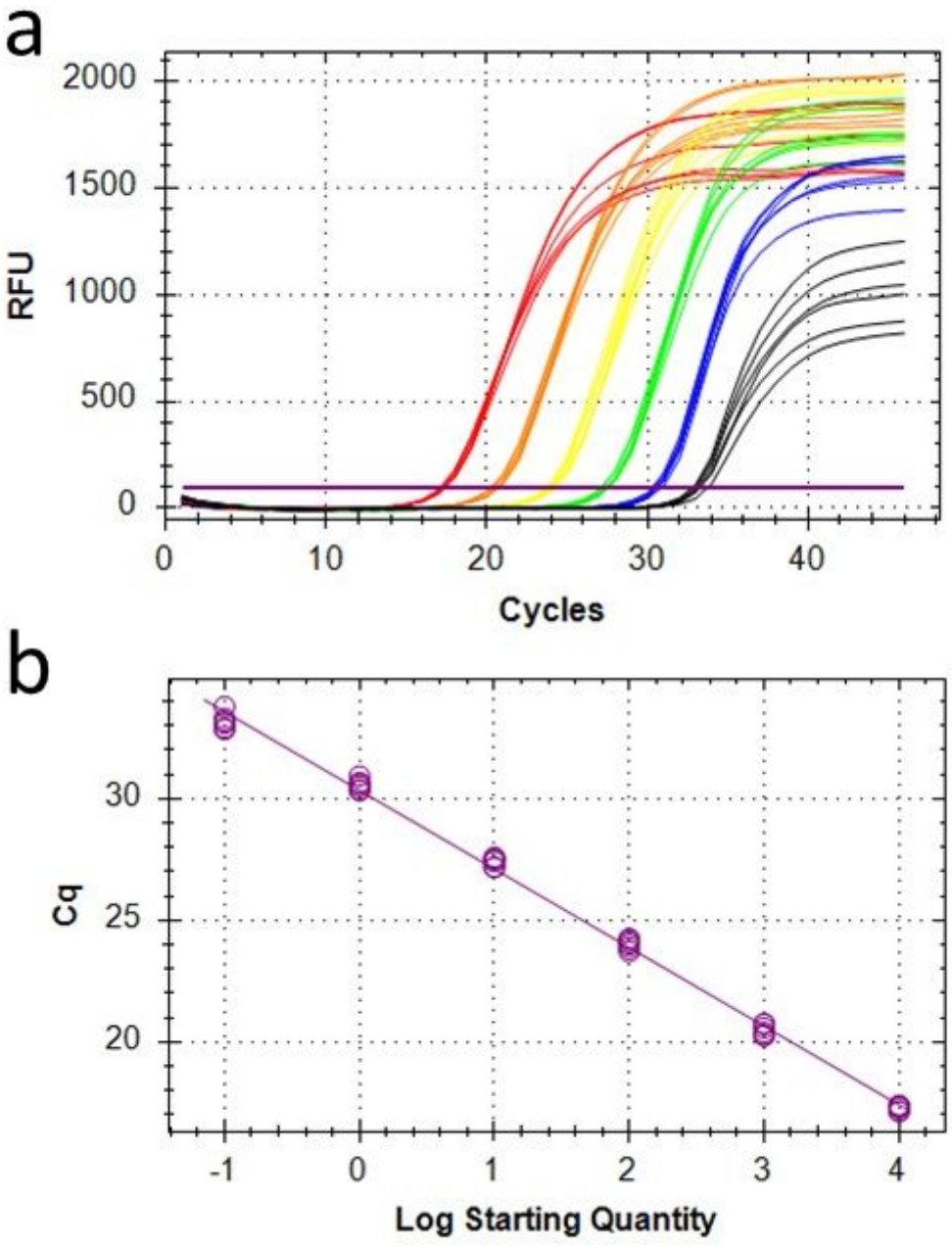

Figure 3

Characteristics of the Hham-qPCR1 with an EGFP internal control. (a) DNA equivalents of 0.1 H. hammondi oocysts reacted positive in the PCR (colour represent results for different DNA equivalents: red, 104; orange, 103; yellow, 102, green, 10; blue, 1; black, 0.1 oocysts). (b) The Hhamq-PCR1 was characterized by a PCR efficiency of E=103.9% and linear regression using DNA equivalents of 104 – 10-1 oocysts yielded a coefficient of determination (R2) of 99.7%.

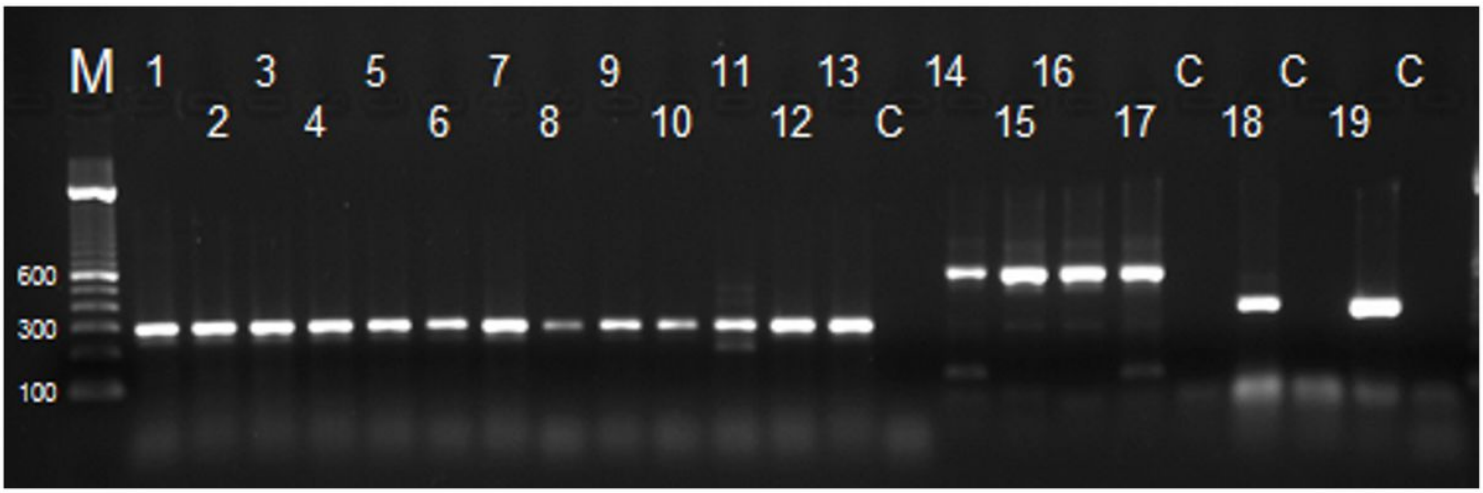

Figure 4

Confirmation that samples used to determine the analytical specificity of HhamqPCR contained sufficient parasite DNA. Lanes 1-19: H. hammondi, T. gondii (RH), T. gondii (ME49), T. gondii (NED), N. caninum (Nc1), B. besnoiti (Evora), H. heydorni, Eimeria bovis, Isospora felis, I. rivolta, I. burrowsi, I. canis, Cryptosporidium parvum, Sarcocystis hirsuta, S. bovifelis. S. hominis, S. cruzi, Giardia spp., Tritrichomonas foetus. DNA in lanes 1-13 had been amplified with Coccidia-specific primer COC1 and COC2, in lane 14-17 with the Sarcocystis spp.-specific primers SarcoFint and SarcoRint, in lane 18 by the Giardia spp.-specific primers RH11_RH4 and RH11_RH5, and in lane 19 by the Trichomonas spp.-specific primers TRF1 and TRF2. M, 100 bp marker, C, no template control.

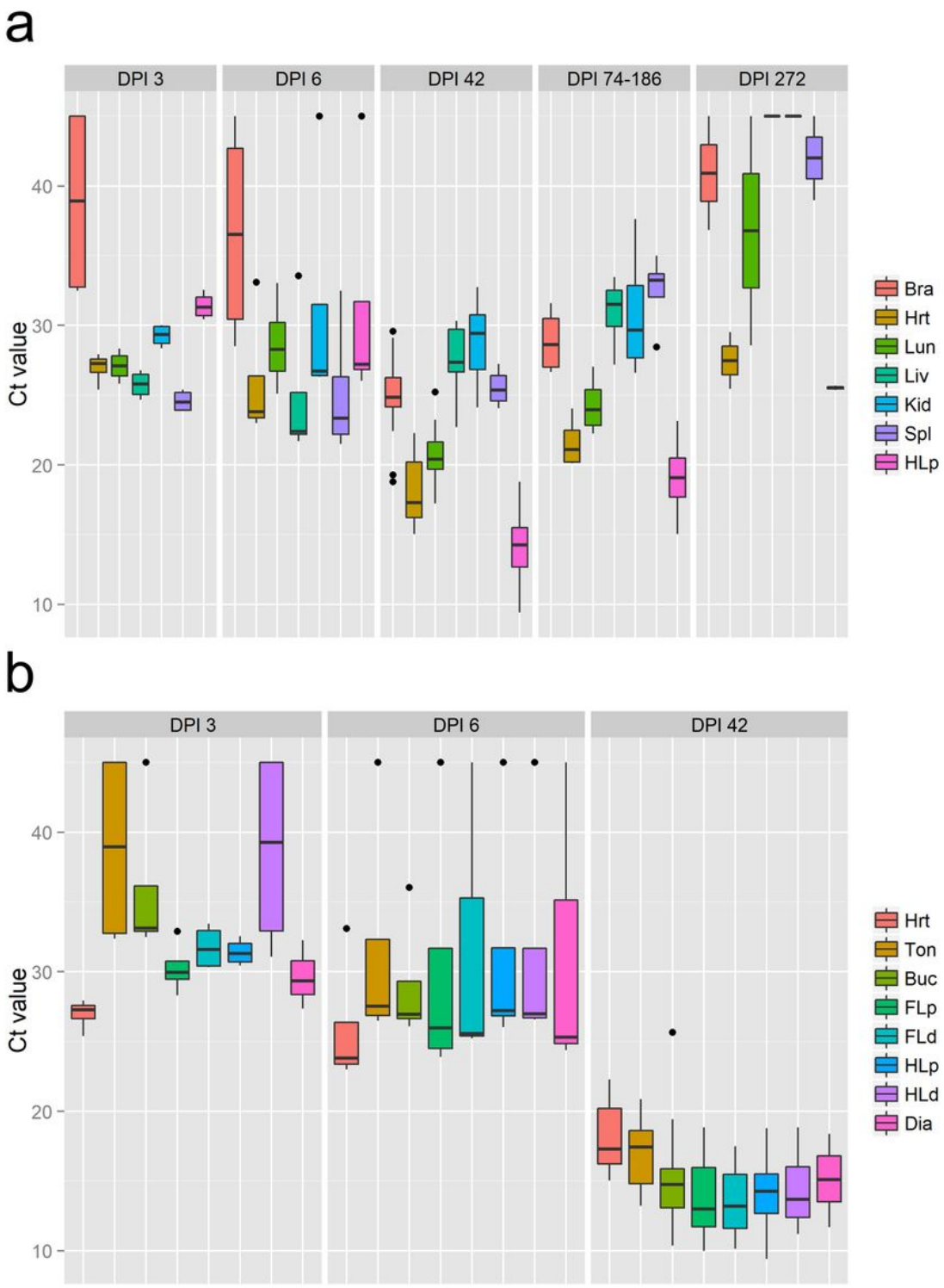

Figure 5

Hham-qPCR1 Ct values in various tissues of IFN-□ knockout (GKO) mice (C.129S7(B6)-Ifngtm1Ts/J) inoculated with Hammondia hammondi. (a) For 28 mice necropsied on days 3, 6, 42, 74-186, or 272 post infection (DPI), seven tissues, brain (Bra), heart (Hrt), lung (Lun), liver (Liv), kidney (Kid), spleen (Spl) and the proximal hind limb musculature (HLp) were available. (b) For some of the mice, samples of the

proximal hind limb musculature (HLp), tongue (Ton), buccal musculature (Buc), forelimb proximal (FLp), forelimb distal (FLd), hind limb distal (HLd) and diaphragm (Dia) were also available and tested.

Supplementary Files

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