

A quantitative-PCR based method to estimate ranavirus viral load following normalisation by reference to an ultraconserved vertebrate target



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ABSTRACT

Ranaviruses are important pathogens of amphibians, reptiles and fish. To meet the need for an analytical method for generating normalised and comparable infection data for these diverse host species, two standard-curve based quantitative-PCR (qPCR) assays were developed enabling viral load estimation across these host groups. A viral qPCR targeting the major capsid protein (MCP) gene was developed which was specific to amphibian-associated ranaviruses with high analytical sensitivity (lower limit of detection: 4.23 plasmid standard copies per reaction) and high reproducibility across a wide dynamic range (coefficient of variation below 3.82% from 3 to 3×10^8 standard copies per reaction). The comparative sensitivity of the viral qPCR was 100% ($n = 78$) based on agreement with an established end-point PCR. Comparative specificity with the end-point PCR was also 100% ($n = 94$) using samples from sites with no history of ranavirus infection. To normalise viral quantities, a host qPCR was developed which targeted a single-copy, ultra-conserved non-coding element (UCNE) of vertebrates. Viral and host qPCRs were applied to track ranavirus growth in culture. The two assays offer a robust approach to viral load estimation and the host qPCR can be paired with assays targeting other pathogens to study infection burdens.

1. Introduction

Ranaviruses (genus *Ranavirus*; family *Iridoviridae*) are large double stranded DNA viruses with broad host ranges which can cause systemic disease in ectothermic vertebrates (Chinchar, 2002). The genus has been divided into amphibian-associated ranaviruses (AARVs) – previously, frequently referred to as amphibian-like ranaviruses (Jancovich et al., 2010; Price, 2016) – and the fish associated Santee-Cooper ranaviruses and grouper iridoviruses (GIV-like) based on phylogenetics (Jancovich et al., 2015). The AARVs comprise three major groups: the frog virus 3 (FV3)-like, common midwife toad virus (CMTV)-like, and *Ambystoma tigrinum* virus (ATV)-like viruses (Jancovich et al., 2015; Price, 2016). AARVs have been repeatedly associated with disease in amphibian hosts but can also infect reptiles and fish (Stöhr et al., 2015).

Ranavirus infection of amphibians and epizootic haematopoietic necrosis disease in fish are both listed as notifiable diseases by the World Organisation for Animal Health (OIE). The OIE currently

includes among others, cell culture, ELISA, and end point PCR as recommended tests for ranavirus detection (OIE, 2014). Despite not being included on this list, quantitative PCR (qPCR)-based methods are commonly used as a screening tool for ranavirus (Black et al., 2017).

Quantitative PCR is considered a sensitive and precise technology with a wide dynamic range (Caraguel et al., 2011; Klein, 2002) but the major technical advantage is the ability to generate quantitative data in contrast to the mostly qualitative data produced by endpoint PCR. This is a powerful attribute as, in the context of infectious disease, it allows the quantification of an infection, its progression, and any tissue tropism. This quantitative element is left underutilised in the absence of appropriate normalisation steps to control for variations in the amount and type of tissue sampled, efficiencies of different nucleic acid extraction methods, and final elution volumes used (Holopainen et al., 2011; Jabs et al., 2001).

Normalisation can be applied by equalising the amount of starting material (e.g. body fluid or tissue) used for nucleic acid extraction, and/or by reporting pathogen genome copies per millilitre or per gram of

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sample used for DNA extraction. This however, does not account for variability in extraction efficiencies and precludes comparisons between tissue types with varying cell densities.

Previously, ranavirus qPCR data has been normalised by total DNA mass e.g. the concentration of template DNA is normalised across samples prior to qPCR analysis (Gray et al., 2009; Hoverman et al., 2010) but this method also has its limitations as 1) it assumes that all DNA is host derived, 2) it relies on precise photometric quantification of DNA, and 3) it is unsuitable for inter-species viral load comparisons when host genome size varies.

It is possible to overcome these confounding effects by normalising by host cell quantity. This can be estimated using a qPCR assay that targets a single-copy host gene in the nuclear genome (Jabs et al., 2001). The use of a second assay targeting a host gene alongside the pathogen assay has the additional benefit of serving as an internal positive control for DNA extraction success and indicates the absence of PCR inhibitors (Dale et al., 2016). Holopainen et al. (2011) developed a qPCR assay targeting the glucokinase (GK) gene of common carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*) to normalise ranavirus qPCR data by host cell quantity in these fish species. Jabs et al. (2001) also used normalisation by reference to a host gene to quantify cell-associated Epstein-Barr virus and found that viral loads generated in this way increased the diagnostic value of the assay in comparison to normalisation by photometrically-determined DNA mass.

Ranaviruses are increasingly recognised as important and emerging multi-host pathogens with very broad host ranges (Gray et al., 2009). Consequently, there is a growing need for analytical methods that permit comparison of infection burdens between 1) the diverse susceptible host species, and 2) diverse sample types (in terms of the amount and type of tissue sampled, and the DNA extraction protocol used).

Due to the very broad host range of ranaviruses, for ranavirus qPCR data to be normalised by host cell quantity, the normalising host target would have to be highly conserved between diverse species. Ultraconserved non-coding elements (UCNEs) are non-coding regions, more than 200 base pairs (bp) in length that share ultra-high sequence identity among vertebrates (Dimitrieva and Bucher, 2013). These are promising candidate targets for a broadly applicable normalising host-qPCR because: 1) they are highly conserved, allowing the design of minimally degenerate primers and probe; 2) they are encoded in the nuclear genome (as opposed to mitochondrial) meaning that, assuming the sequence is present at a single copy in the haploid genome, target copy number equates to an approximate cell quantity; 3) they are of sufficient length to enable qPCR primer and probe design; and 4) they are only present in vertebrates (Bejerano et al., 2004; Retelska et al., 2007) minimising the risk of non-specific amplification due to non-host DNA present in a sample, such as from bacteria, fungi, or parasites.

This study describes the development of two qPCR assays: one to detect AARVs by targeting a conserved region of the MCP gene, and a second qPCR assay targeting a single-copy UCNE of vertebrates, which serves to quantify host cells. The qPCRs can be used together to generate viral load estimates for comparison of AARV infections in amphibian, reptile, and fish hosts. The method is described here along with a demonstration of its analytical specificity and sensitivity, its comparative specificity and sensitivity, and an application.

2. Methods

2.1. Design and optimisation of viral and host qPCR protocols

2.1.1. Target selection

The ranavirus MCP was targeted as a conserved, virus-specific gene for which there was abundant sequence data from diverse viruses in GenBank (Clark et al., 2016). In order to normalise virus quantity, a second qPCR was designed to quantify a single-copy host target as a proxy for host cell quantity.

UCNEbase is an online database containing human UCNE sequences and their orthologs in eight other species (Dimitrieva and Bucher, 2013). Human UCNEs with greater than 90% sequence identity to *Xenopus tropicalis* (the only amphibian species in the database) were downloaded from UCNEbase. Straightforward and precise normalisation of virus quantity by a host target requires that copy number is consistent between species so copy number was assessed for candidate UCNEs. Candidate sequences were used as queries in homology searches (BLASTn searches with default settings except max e-value which was set to 0.001; McGinnis and Madden, 2004) against a panel of 64 vertebrate genomes (including one amphibian, two reptiles and 12 fish; Table S1) and five invertebrate species in the Ensembl genome browser database (Yates et al., 2016). Ectothermic vertebrate classes with susceptibility to ranavirus (amphibians, reptiles and fish) were the main groups of interest but other species were included in these analyses to get a broader view of copy number across species. Candidate UCNEs were retained if the following conditions were met: 1) single hits were returned for more than 90% of vertebrates suggesting that a specific qPCR assay could be designed and the sequence was present as a single copy in the haploid genome; 2) no hits to invertebrate species were returned.

2.1.2. Primer and probe design

Both qPCRs were designed as hydrolysis probe-based methods. Primers and probe were designed using Primer3 version 4.0.0 (Koressaar and Remm, 2007; Untergasser et al., 2012) using search parameters described by D'haene et al. (2010). To design a ranavirus assay which targeted AARVs, a consensus sequence was made from viral sequences representing the major groups of AARV (FV3 (GenBank accession number AY548484; Tan et al., 2004)), tiger frog virus (TFV; AF389451; He et al., 2002), CMTV (JQ231222; Mavian et al., 2012) and epizootic haematopoietic necrosis virus (EHNV; FJ433873; Jancovich et al., 2010); i.e. Santee Cooper ranaviruses and GIV-like ranaviruses were excluded. For the host qPCR, the region of EBF3_Napoleon (UCNEbase id = 8107) from bases 159–242 (referred to as EBF3N) which aligned to 60/64 vertebrate genomes was used as input for Primer3. Hydrolysis probes with 3' MGB-NFQ quenchers and 5' VIC or 6FAM reporters were used for the viral (MCP) and host (EBF3N) assays respectively (Table 1).

2.1.3. Producing standards

To enable absolute quantification of viral or host DNA by standard

Table 1

Primer and probe sequences for all qPCR assays used. The viral qPCR targets the ranavirus major capsid protein gene (MCP). The host qPCR targets the ultra-conserved non-coding element, EBF3_Napoleon (EBF3N). Recombination activating gene 1 (RAG1) was used as a known single copy gene to assess copy number of EBF3N. IUPAC ambiguity codes are used to denote degenerate bases.

Primer/probe name	Nucleotide sequence ^a 5'→3'
MCP_F	GTCTTTAAGACGGCATACT
MCP_R	ATCGCTGGTGTGCCTATC
MCP_probe ^b	TTATAGTAGCCTRTGCGCTTGGCC
EBF3N_F	ATGCTGCAATTCAAACTGTCCAG
EBF3N_R	CAGTAAGCAAATKGGGAAGAAGC
EBF3N_probe ^c	CACCTGGTTTGTCTCAGGGATA
Frog_RAG1(a)_F	ACAGCCATCTTGAGCCCTTT
Frog_RAG1(a)_R	TACCCTGTGCCACGAAAGAC
Frog_RAG1(b)_F	TTGCTGCCAGGGTATCATCC
Frog_RAG1(b)_R	CAGTCTGAAAGCCCATCCA
Toad_RAG1(a)_F	AAGAGTGCCAGAGTTGCTC
Toad_RAG1(a)_R	GTGGCTAGGGTTTGTGGA
Toad_RAG1(b)_F	CACGGTGAGAACTTGCAACG
Toad_RAG1(b)_R	TGGCTTTGCAGAACTCCCT

^a IUPAC codes: R = A/G; K = G/T.

^b 5' VIC reporter, 3' MGB-NFQ quencher; fits anti-sense strand.

^c 5' FAM reporter, 3' MGB-NFQ quencher; fits sense strand.

curve, plasmid standards containing the viral MCP target (amplified from an FV3-like virus isolate, RUK13; KJ538546; Price, 2014), and the host EBF3N target (amplified from *Rana temporaria*; common frog), were constructed using TOPO TA Cloning Kit (ThermoFisher Scientific). Colonies were selected by blue/white screening, expanded in 50 mL Luria broth with 50 µg/mL ampicillin, and harvested using the PureYield Plasmid Midiprep System (Promega). To confirm the presence of inserts, plasmid constructs were sequenced along both strands using universal M13 primers (Beckman Coulter Genomics). Each construct was then linearised by digestion with the EcoRV-HF restriction enzyme (New England Biolabs; 5 µL EcoRV-HF at 20 units/µL, 10 µL 10 × NEBuffer, and 85 µL of each plasmid at 50 ng/µL) incubated at 37°C for 16 h, and followed by 65°C for 20 min. Complete digestion was confirmed by agarose gel electrophoresis using undigested plasmid as a control.

A set of ten viral and host qPCR standards were then prepared with concentrations of 3×10^9 to 3 copies per 2 µL using 10-fold serial dilutions in nuclease-free water. First, the mass of a single viral or host plasmid construct was calculated given that a) the mass of 1 bp = 1.096×10^{-21} g and b) the pCR2.1 TOPO plasmid backbone = 3931 bp, the MCP insert = 97 bp, the EBF3N insert = 104 bp. The concentrations of each plasmid stock were then quantified in triplicate using a Qubit 2.0 Fluorometer and the dsDNA BR Assay Kit (Thermo Fisher Scientific) and mean concentrations were calculated. These values were then used to dilute each plasmid stock to 3×10^9 copies per 2 µL.

Only a single amphibian genome (*X. tropicalis*) was used in the in silico assessment of host targets (Table S1; 2.1.1 Target Selection). To confirm that EBF3N exhibited ultra-high level conservation across amphibian species and therefore ensuring that the primer and probe sequences would anneal to these templates, the EBF3N target region was also cloned and sequenced for *Bufo bufo* (common toad) and *Ichthyosaura alpestris* (alpine newt) using the same methods described above for *R. temporaria*. DNA derived from a single animal from each species was used as template for PCRs. Two clones were selected and sequenced for each individual.

2.1.4. Viral load estimation using viral and host qPCRs

MCP and EBF3N qPCRs were run independently in single-plex qPCR reactions throughout the entirety of the study. Duplicate reactions of each sample were run on PCR plates which included two no template controls (NTCs) and a set of duplicate standards sufficient to generate a standard curve spanning the copy number range observed in “unknown” samples. Standard curve calculations were initially based on standard dilution series in the range of 3– 3×10^9 target copies per 2 µL but later restricted to three standards (3×10^7 , 3×10^4 and 3×10^1 target copies per 2 µL) once a typical range for unknown samples was established.

Reactions were set up in 20 µL total volumes comprising 10 µL TaqMan Universal 2 × PCR Master Mix (ThermoFisher Scientific), 5.95 µL nuclease free water, 0.5 µM each primer, 250 nM probe, and 2 µL template DNA. Reactions were loaded onto 0.1 mL MicroAmp Optical 96-Well Reaction Plates (ThermoFisher Scientific) and sealed with MicroAmp Optical Adhesive Film (ThermoFisher Scientific). Thermocycling was performed using the StepOnePlus Real-Time PCR System (ThermoFisher Scientific) and the following settings: 50 °C for 2 min [Uracil-N glycosylase activation to prevent carry-over contamination by PCR products (Longo et al., 1990)], 95 °C for 10 min, and 50 cycles of 95 °C for 15 s and 60 °C for 30 s. Samples were considered positive if a sigmoidal amplification curve was present which accumulated fluorescence above the threshold cycle (CT) in both replicates and negative otherwise. Runs were considered valid if 1) the CT values fell within the range covered by the standards, 2) all NTCs were negative, and 3) PCR efficiency was between 85% and 100%.

Data were analysed in the StepOne software (v2.0). The baseline was set automatically and threshold was set manually at the point

where all amplification traces were in their log phases. Where multiple samples spanning multiple runs were analysed together, this threshold was kept constant so that CT values were comparable.

2.1.5. Viral load calculation

Normalisation of viral MCP quantity by host EBF3N quantity returns an effective viral load i.e. MCP copies per cell. Eq. (1) was used to correct for ploidy of a given organism, particularly important for amphibians since polyploidy is not uncommon (Evans et al., 2005).

$$\text{Viral load (MCP copies per host cell)} = \frac{\text{MCP quantity} \times \text{ploidy}}{\text{EBF3N quantity}} \quad (1)$$

2.2. Protocol assessment

2.2.1. In silico specificity

The specificity of primer pairs for intended targets can be assessed using Primer-Blast by performing similarity searches of sequence databases for unintended targets according to stringency parameters with any combination of the primers (forward-reverse, forward-forward, reverse-reverse) (Ye et al., 2012). The primer pairs for both the viral and host qPCRs were checked for specificity using Primer-Blast and parameter settings relaxed to increase the capacity to detect possible unintended targets. Primer-Blast did not allow use of ambiguity codes within search queries so separate searches were conducted to accommodate degeneracy in the EBF3N reverse primer. All searches were conducted against the GenBank ‘nr’ database with expect value cut-off increased to 100,000, word size decreased to six, the maximum number of database sequences increased to 100,000 and maximum target length set to 4000. Default settings for parameters which handled mismatches with primer sequences were used: mismatch tolerance threshold (above which targets were ignored) set to six with a maximum of two mismatches falling within the final five bases at the primer’s three-prime end.

To rapidly assess relatedness among Primer-Blast hits generated with the viral qPCR primers, the sequences for the final lists of filtered hits were downloaded using a batch query to Entrez on their accession numbers. The sequences were combined with an MCP sequence from grouper iridovirus (accession JF264365; Huang et al., 2011) – considered an outgroup to AARVs (Price, 2016) – prior to alignment with Mafft v7.130b using the automatic, accurate direction adjustment function. The alignments were edited in Jalview (Waterhouse et al., 2009) to remove columns with gaps and then used to calculate summaries of pairwise genetic distances using the default Kimura’s 2-parameter distance model in the R package, Ape (Paradis et al., 2004).

To summarise taxonomic information relating to hits generated with the host qPCR primers, species names were extracted from the Primer-Blast output and were used to retrieve the classification hierarchy for each from the Integrated Taxonomic Information System database using the R package taxize (Chamberlain and Szöcs, 2013).

2.2.2. Assessment of EBF3N copy number using the $2^{-\Delta\Delta CT}$ method

In silico analyses suggested that EBF3N was present as a single copy gene across all tested vertebrate species. To further test for the absence of within-species copy number variation, comparisons were made between quantities of EBF3N in multiple individuals of two species, *R. temporaria* and *B. bufo*. Assessment of copy number variation was determined using relative quantification for qPCR and the comparative CT method (the $2^{-\Delta\Delta CT}$ method; Livak and Schmittgen, 2001). This method determined gene copy number using a known single-copy gene as the comparator (Ma and Chung, 2014; D’haene et al., 2010). Recombination activating gene 1 (RAG1) is known to be single-copy across amphibian species (Chiari et al., 2009; Evans et al., 2005) and was used as the comparator for assessment of EBF3N copy number. Two comparator qPCR assays, each targeting a different region of RAG1, were used to increase the accuracy and reliability of results (D’haene

et al., 2010).

RAG1 primers were designed against sequences from *R. temporaria* (AY323776.1) and *B. bufo* (AY323762.1) following the primer design methods described previously. Each 15 µL reaction consisted of 7.5 µL 2x GoTaq qPCR Master Mix containing BRYT Green (Promega), 0.75 µL of each primer at 10 µM, 0.375 µL CXR reference dye, 4.125 µL nuclease-free water and 1.5 µL template DNA. Thermocycling was performed using instrumentation and consumables described previously and the following cycle settings: 95 °C for 2 min; 40 cycles of 95 °C for 15 s, 60 °C for 60 s followed by a melt curve stage from 60 °C to 95 °C, increasing by 0.3 °C every 15 s. PCR efficiencies of all assays were similar. Specificity of all assays was confirmed by the presence of single melt curves for all PCR reactions.

The three targets – RAG1(a), RAG1(b), and EBF3N – were quantified for seven *R. temporaria* and seven *B. bufo* individuals using the in-built comparative CT method in the StepOne software (v.2.0). EBF3N was selected as the endogenous control and one of the seven animals was selected as a ‘reference’ individual. Relative quantity (RQ) values for each RAG1 locus [RAG1(a) and RAG1(b)] were generated for all six ‘test’ individuals of each species. Since RAG1 is single-copy, an RQ of 1 would be expected if EBF3N was also single-copy. Minimum and maximum RQ values were calculated automatically by incorporating the standard deviation of the $\Delta\Delta\text{CT}$ value (calculated from the standard deviations of the duplicate qPCR reactions) into the RQ calculation. In the absence of a calibrator sample in which EBF3N copy number was known, this analysis was limited to the detection of copy number variation between the tested animals. Variation in RAG1(a) and RAG1(b) RQ values was assessed using one-way ANOVA.

2.2.3. Analytical specificity

To experimentally assess the analytical specificity of the viral qPCR, the assay was run using template DNA from known AARVs extracted from amphibian tissues: FV3, CMTV, Ándaran *Alytes obstetricans* virus (AAOV), Bosca's newt virus (BNV), and ATV. DNA extracted from cultured Scale Drop Virus (SDV), a member of the genus *Megalocytivirus* which is also part of the family *Iridoviridae* (De Groof et al., 2015), served as a negative control for specificity against AARVs. Template DNA extracted from uninfected tissues of the amphibians, reptiles, and fish listed in Table 2 served as further negative controls of specificity for the viral qPCR.

For the host qPCR, DNA extracts from eight amphibian species spanning the Anura and Caudata, four reptiles, and three fish (Table 2)

Table 2

List of species used to experimentally assess specificity of the viral and host qPCRs.

Group	Species	Common name	Family
Amphibian	<i>Bufo bufo</i>	Common toad	<i>Bufo</i> idae
Amphibian	<i>Rana temporaria</i>	Common frog	<i>Rana</i> idae
Amphibian	<i>Pelophylax perezi</i>	Perez's frog	<i>Rana</i> idae
Amphibian	<i>Andrias davidianus</i>	Giant Chinese salamander	<i>Cryptobranchiidae</i>
Amphibian	<i>Triturus marmoratus</i>	Marbled newt	<i>Salmandridae</i>
Amphibian	<i>Hyla molleri</i>	European tree frog	<i>Hylidae</i>
Amphibian	<i>Lissotriton boscai</i>	Bosca's newt	<i>Salmandridae</i>
Amphibian	<i>Alytes obstetricans</i>	Common midwife toad	<i>Alytidae</i>
Reptile	<i>Varanus komodoensis</i>	Komodo dragon	<i>Varanidae</i>
Reptile	<i>Natrix maura</i>	Viperine water snake	<i>Colubridae</i>
Reptile	<i>Podarcis bocagei</i>	Bocage's wall lizard	<i>Lacertidae</i>
Reptile	<i>Iguana iguana</i>	Green iguana	<i>Iguanidae</i>
Fish	<i>Salmo salar</i>	Atlantic salmon	<i>Salmonidae</i>
Fish	<i>Scomber scombrus</i>	Atlantic mackerel	<i>Scombridae</i>
Fish	<i>Merluccius merluccius</i>	European hake	<i>Merlucciidae</i>
Bacteria	<i>Serratia liquefaciens</i>	N/A	<i>Enterobacteriaceae</i>
Bacteria	<i>Pseudomonas azotoformans</i>	N/A	<i>Pseudomonadaceae</i>
Bacteria	<i>Aeromonas media</i>	N/A	<i>Aeromonadaceae</i>
Bacteria	<i>Acinetobacter</i> spp.	N/A	<i>Moraxellaceae</i>

were used as PCR template. DNA samples from pure cultures of bacteria isolated from amphibian skin were used as negative controls of specificity (Table 2).

2.2.4. Analytical sensitivity

The analytical sensitivity of each qPCR assay was defined by its lower limit of detection (LoD) which was in turn defined as the lowest copy number per PCR reaction that produced a positive result 95% and 100% of the time. Two-fold serial dilutions of each standard were made to give 15, 7.5, 3.75, and 1.875 target copies per 2 µL. At each dilution, 24 replicate reactions were run on a single 96-well PCR plate. The dilution that resulted in 95% (22.8 reactions) of positive results was calculated using logistic regression in R (R Core Team, 2013).

2.2.5. Assay performance compared to an established end-point PCR

The method described by Mao et al. (1997) is the most commonly used endpoint PCR for ranavirus detection (Black et al., 2017). Where infection status was determined by the Mao et al. (1997) endpoint PCR, the capacity of the viral qPCR to detect ranavirus in infected animals (termed comparative sensitivity) and to correctly differentiate between infected and uninfected animals (termed comparative specificity) was assessed.

To measure comparative sensitivity, 78 DNA samples from wild amphibians (72 *R. temporaria*, 6 *B. bufo*, all adults) which tested positive for ranavirus using the Mao et al. (1997) PCR and had been confirmed by Sanger sequencing were tested with the viral qPCR. To measure comparative specificity, 94 live wild amphibians (1 *Alytes obstetricans*, 20 *Hyla molleri*, 33 *Ichthyosaura alpestris*, 23 *Pelophylax perezi*, 17 *Salamandra salamandra*, all larvae or juveniles) originating from sites with no history of ranavirus infection and which tested negative for ranavirus using the Mao et al. (1997) PCR, were also tested with the viral qPCR. DNA was extracted from the livers of moribund animals and from toe/tail clips of live animals using DNeasy Blood & Tissue Kit (Qiagen) and following the manufacturer's instructions.

2.2.6. Reproducibility, PCR efficiency, and linear dynamic range

Measures of intra- and inter-assay variation were used to assess assay reproducibility. The plasmid standards containing $3\text{--}3 \times 10^9$ target copies/reaction were used as template DNA. For both qPCR assays, each dilution point was run in triplicate within a single run to assess intra-assay variation. To assess inter-assay variation, the same plate setups were repeated across three separate runs resulting in nine replicates in total (three replicates on each of three plates). Mean CT scores were calculated and plots of CT against target number were used to determine the linear dynamic range of each assay. PCR efficiencies were determined using the formula: $E = (10^{-1/\text{slope}} - 1) \times 100$. Assay variation was measured as CT coefficients of variation at each dilution point. Thresholds were standardised at 0.04 ΔRn for MCP and 0.15 ΔRn for EBF3N to allow comparison between plates.

2.2.7. Quantification of viral growth in cell culture

To demonstrate an application of the combined use of the viral and host qPCRs, the method was used to track ranavirus growth in cell cultures over time. A single 24-well cell culture plate (VWR) was seeded with 1×10^6 IgH-2 cells (iguana heart 2; ECACC 90030804) in 500 µL of cell culture media per well (Eagle's minimum essential medium [EMEM] base media with Earle's balanced salt solution [EBSS] + 2 mM L-Glutamine + 1% non-essential amino acids + 10% foetal bovine serum [ThermoFisher Scientific]). Cells were incubated for two days at 26 °C, 5% CO₂ to allow them to adhere to the well surface. Twenty-two wells were inoculated with 100 µL of ranavirus (isolate RUK13) containing a total of 50,000 viral genome copies (quantified with the viral qPCR) and the remaining two wells were inoculated with 100 µL culture media as negative controls. The plate was incubated at 24 °C and duplicate wells were harvested at 1, 4, 8 and 24 h, and then daily until 8 days post infection (p.i.). Control wells were also harvested on day 8.

Upon harvest, the media and the cells were separated: media was depleted of cells by centrifugation at 1000g for five minutes. The adherent cells were harvested by incubating with 200 μ L Trypsin-EDTA (0.25%) for three minutes, followed by neutralisation with an equal volume of cell culture media and then centrifugation at 1000g for five minutes. For each well, the cell pellets from adherent and suspended cells were then combined. All samples (cell pellets containing cell-associated virus and media containing extracellular virus) were stored at -20°C until the end of the experiment and extracted together using DNeasy 96 Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions. To extract DNA from the cell culture media, the protocol for non-nucleated blood was followed using 200 μ L harvested media. All samples were eluted in 200 μ L elution buffer.

The viral qPCR was used to quantify virus in the 'media' and 'cell' samples. The host qPCR was used to quantify total cell number in the cell sample and this value was used to calculate viral load in IgH-2 cells. Target quantities were scaled to account for sample volumes. All sample values were multiplied by 100 to adjust to total volume eluted (2 μ L only of 200 μ L total eluted volume was used as template in qPCR reactions) and viral quantities from media were also multiplied by 3 (to adjust the volume extracted [200 μ L] to the total volume of media in a well [600 μ L]).

3. Results

3.1. Design and optimisation of viral and host qPCR protocols

3.1.1. Target selection

Homology searches using EBF3N as the query sequence returned single hits for 60/64 vertebrates including 1/1 amphibians, 2/2 reptiles, 12/12 fish, and 45/49 other vertebrates. 4/64 vertebrates (all endothermic) and 5/5 invertebrates returned no hits. All alignments covered the full 84 bp of the query sequence except *Astyanax mexicanus* (cave fish) where the alignment was only 56 bp in length.

3.1.2. Sequencing

EBF3N sequences for *R. temporaria*, *B. bufo*, and *I. alpestris* shared $\geq 98.8\%$ sequence identity with UCNEbase sequence 8107 from bases 159–242 (Fig. 1B). The 1.2% miss-match was due to an apparent single nucleotide polymorphism (SNP) in a region covered by the reverse primer with at least two alleles. For each individual from each species, the two sequenced clones revealed that all individuals were heterozygous for this apparent SNP (C/A). This variation was accounted for by incorporating a single degenerate base in the reverse primer (G/T – International Union of Pure and Applied Chemistry [IUPAC] ambiguity code, K).

3.1.3. Primer and probe design

Through the incorporation of a single degenerate base (A/G – IUPAC ambiguity code, R) in the probe sequence, viral qPCR primers and probe shared 100% complementarity to the template viruses (FV3, TFV, CMTV, EHNV) (Fig. 1A). The EBF3N primers and probe had 100% complementarity to all the amphibian and reptile species and 9/12 fish species that were assessed. The fish species with mismatches were *Astyanax mexicanus* (cave fish), *Danio rerio* (zebrafish), and *Gadus morhua* (cod); the latter appeared to have a single base pair deletion at base 39 of EBF3N (Fig. 1B). Of the other vertebrate species with single hits for EBF3N, 26/45 had 100% complementarity to the EBF3N primers and probe sequences.

3.2. Protocol assessment

3.2.1. Assessment of EBF3N copy number using the $2^{-\Delta\Delta\text{CT}}$ method

The average relative quantity (RQ) of RAG1(a) and RAG1(b) to EBF3N was 1.05 (standard deviation = 0.09) and 1.02 (standard deviation = 0.1) respectively for *R. temporaria*, and 0.96 (standard

deviation = 0.09) and 0.96 (standard deviation = 0.10) for *B. bufo*. Variation in RQ values were not significantly different between RAG1(a) and RAG1(b) from the two species ($F(3,20) = 1.31$, $p = 0.298$), consistent with EBF3N being devoid of copy number variation in these species.

3.2.2. In silico specificity

The viral qPCR primers returned 6478 blast hits, of which 145 passed target filtering. All hits had a target length matching the expected target size (97 bp). After alignment of hits to grouper iridovirus, the sequence of accession GU292010, which is considered an AARV (Weir et al., 2012), was removed due to poor overlap with the remaining sequences. All positions with gaps were then removed resulting in a final alignment of 145 sequences and 381 nucleotides. The mean pairwise genetic distance among hits (excluding grouper iridovirus) was 0.022 (standard deviation = 0.014, range = 0–0.069). As a comparison, the mean distance of grouper iridovirus to the hits was 0.35 (standard deviation = 0.0072, range = 0.33–0.37). The high sequence identity among hits in contrast to their distance to the non-AARV out-group indicated that the viral qPCR primers had returned only hits to AARVs, and had therefore delivered the desired specificity.

The Primer-Blast search with the host qPCR primers considered 84,389 Blast hits but only 74 passed filtering. All but three of these matched the expected target size (84 bp) and were from vertebrates (64 fish, 1 amphibian, 1 bird, 5 mammals). The remaining three hits corresponded to two unintended targets which, as well as containing multiple mismatches to the primers, were too large to amplify by qPCR (2198 and 1174 bases in length).

3.2.3. Analytical specificity

The viral qPCR detected all ranaviruses tested, which included members of each of the major groups of the amphibian-associated ranaviruses. The reactions containing template DNA from uninfected individuals of four host species and SDV were all negative demonstrating an absence of non-specific amplification with host and non-target viral DNA from a member of another genus in the same virus family. The host qPCR detected all eight amphibian, four reptile, and three fish species. Negative controls containing bacterial DNA were all negative.

3.2.4. Analytical sensitivity

The 95% LoD values of the viral and host qPCRs were 4.23 and 3.26 standard copies per reaction respectively. The lowest copy number detected 100% of the time was 7.5 standard copies per reaction for both assays.

3.2.5. Assay performance compared to an established end-point PCR

All 78 samples (72 *R. temporaria* and 6 *B. bufo*) that screened positive by endpoint PCR (Mao et al., 1997) also tested positive with the viral MCP qPCR: 100% comparative sensitivity (median CT 21.11; CT range 9.99–40.06; median MCP quantity 4.345×10^6 per 2 μ L; MCP range 1.602×10^1 – 6.618×10^9 per 2 μ L). There was no significant difference in MCP quantity for *R. temporaria* and *B. bufo* [$t(53.0) = 1.11$, $p = 0.27$]. All 94 samples from ranavirus-free sites and which tested negative by endpoint PCR (Mao et al., 1997), tested negative with the viral qPCR: 100% comparative specificity.

3.2.6. Reproducibility, PCR efficiency, and linear dynamic range

The linear dynamic range of the viral qPCR was found to be from 3 to 3×10^8 standard copies per reaction. However, the dilution with 30 target copies/reaction lay just off the line of best fit (Fig. 2A). The average efficiency was 86.66%. The linear dynamic range of the host qPCR, was from 3 to 3×10^9 standard copies per reaction, covering the entire range tested (Fig. 2B). The average efficiency was 99.2%. The coefficient of determination (R^2) was 0.99 for both assays and slopes were -3.69 for the viral qPCR (Fig. 2A) and -3.34 for the host qPCR (Fig. 2B).

A

	FORWARD PRIMER																																																																																					
	1	10	20	30	40	50	60	70	80	90	97																																																																											
RV3	G	T	C	C	T	T	T	A	A	C	A	C	G	G	C	A	T	A	C	C	T	G	G	A	G	C	G	A	G	T	A	C	A	C	C	A	T	G	C	C	A	G																																												
TFV																																																																																						
CMTV																																																																																						
EHNV																																																																																						

B

	FORWARD PRIMER																														PROBE																																								
	1	10	20	30	40	50	60	70	80	90	97																																																												
Western dawed frog (<i>Xenopus tropicalis</i>)	A	T	G	C	T	G	C	A	A	T	T	C	A	A	A	C	T	G	T	C	A	G	C	A	C	T	G	G	T	T	T	G	C	T	C	A	G	G	G	A	T	A																													
Anole lizard (<i>Anolis carolinensis</i>)																																																																							
Chinese softshell turtle (<i>Pelodiscus sinensis</i>)																																																																							
Amazon molly (<i>Poecilia formosa</i>)																																																																							
Fugu (<i>Takifugu rubripes</i>)																																																																							
Japanese medaka (<i>Oryzias latipes</i>)																																																																							
Platyfish (<i>Xiphophorus maculatus</i>)																																																																							
Spotted gar (<i>Lepisosteus oculatus</i>)																																																																							
Stickleback (<i>Gasterosteus aculeatus</i>)																																																																							
Green spotted puffer (<i>Tetraodon nigroviridis</i>)																																																																							
Tilapia (<i>Oreochromis niloticus</i>)																																																																							
Cod (<i>Gadus morhua</i>)																																																																							
Zebrafish (<i>Danio rerio</i>)																																																																							
Coelacanth (<i>Latimeria chalumnae</i>)																																																																							
Cave fish (<i>Astyanax mexicanus</i>)																																																																							
Common frog (<i>Rana temporaria</i>)																																																																							
Common toad (<i>Bufo bufo</i>)																																																																							
Alpine newt (<i>Ichthyosaura alpestris</i>)																																																																							

Fig. 1. Sequence complementarity of primer and probe oligonucleotide sequences used in host and viral quantitative PCRs. Alignments of target regions for (A) the virus major capsid protein gene (MCP), and (B) the vertebrate ultra-conserved non-coding element, EBF3N. Annealing sites of primers and probes are indicated by boxes. The MCP alignment includes representatives of each of the three major groups of amphibian-associated ranaviruses: frog virus 3 (FV3; GenBank accession AY548484), tiger frog virus (TFV; AF389451), common midwife toad virus (CMTV; JQ231222), and epizootic haematopoietic necrosis virus (EHNV; FJ433873). The EBF3N alignment includes all amphibians (n = 1), reptiles (n = 2), and fish (n = 12) in the Ensembl genome browser database. The amphibian EBF3N sequence data generated in this study is highlighted in grey. Conserved bases are denoted with a ':', deletions by a 'X', and gaps in the alignment with a '-'.

Intra-assay variation ranged from 0.15% to 3.81% for the viral qPCR and between 0.02% and 7.72% for the host qPCR. The highest intra-assay variations were between reactions with the lowest target copy numbers (three standard copies per reaction). When this dilution point was omitted, all coefficients of variation for both assays fell below 2.78%. Inter-assay variation was between 0.25% and 2.69% for the viral qPCR and between 0.73% and 4.4% for the host qPCR. Again, the highest coefficient of variation for both assays was at the lowest copy number and when this dilution point was omitted, all coefficients of variation for both assays were below 2.4%, demonstrating that the reproducibility of both assays was very high within and between runs (Figs. 2A and 2B).

3.2.7. Quantification of viral growth in cell culture

In the 'media' sample, the first 4 h p.i. were marked by a decrease in viral quantity consistent with virus entering cells (Fig. 3A1 and A2).

This was followed by an increase in extracellular virus up to 8 d p.i. when a maximum of 1.38×10^8 copies was reached. In the 'cell' sample, virus quantity increased up to 4 d p.i. when it reached a plateau at approximately 1.4×10^9 copies. Between 1 and 7 d p.i., more than 90% of the total viral DNA was inside or associated with cells (Fig. 3A1). Viral load increased by seven orders of magnitude throughout the duration of the experiment: from 3×10^{-3} to 1×10^4 viral genome copies per host cell (Fig. 3B).

4. Discussion

Ranaviruses are important pathogens which are emerging in populations of ectothermic vertebrates and which frequently affect multi-host communities of such species (Gray et al., 2009; Price et al., 2014). While the association between viral load and ranaviral disease is currently not well understood (Gray et al., 2009), there is some evidence

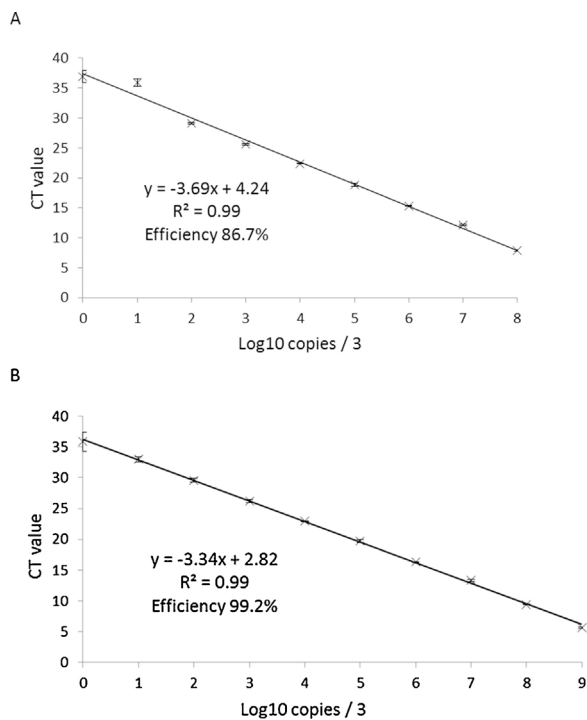


Fig. 2. Standard curves for (A) viral and (B) host qPCR assays. Data points represent averages over nine replicates generated from three separate runs (three replicates per run). Thresholds were standardised between runs to 0.04 and 0.15 for MCP and EBF3N assays, respectively. Error bars show standard deviations of duplicate reactions. Efficiencies were calculated using the formula: $E = (10^{-1/\text{slope}} - 1) \times 100$.

that they are correlated (Hoverman et al., 2010). This work sought to develop a widely applicable tool for ranavirus viral load quantification which could help to elucidate this relationship.

The viral qPCR developed here is analytically highly precise, sensitive, and specific. Analytical specificity was demonstrated through the ability to detect and quantify ranaviruses from all major groups of AARV. Analytical specificity was also demonstrated through an absence of amplification in a series of controls including samples infected by a virus from another genus in the same family, as well as uninfected samples from a broad range of candidate host species. The limit of detection of the viral assay calculated using plasmid standards was close to the theoretical limit of detection of three target copies per PCR reaction (Bustin et al., 2009). The comparative sensitivity of the viral qPCR was 100% relative to the established end point PCR used as the comparator. Comparative specificity was also 100% as the viral qPCR correctly called 100% of ‘known negatives’.

The host qPCR also performed well according to the precision, sensitivity, and specificity criteria. The EBF3N target is a highly conserved single-copy marker across diverse vertebrate species. BLASTn searches of ENF3N against whole genomes found no evidence of interspecies copy number variation in at least 60 vertebrate species including an amphibian, two reptile, and twelve fish species. Intra-species copy number variation was also absent for the two amphibian species that were assessed (*R. temporaria* and *B. bufo*) providing further evidence that EBF3N is a suitable target for normalisation. As such, the host qPCR can be used in combination with the viral qPCR for straightforward comparison of infection intensities among host species. The host qPCR was applicable across species: the single set of primers and probe were able to amplify with template DNA from amphibian, reptile and fish tissues. The nucleotide sequence of our target region (EBF3N) was also shown to be highly similar across amphibian, reptile, and some fish species. The primer and/or probe sequences would, however, require modification prior to use with some fish species due to the lower degree of UCNE sequence conservation in this class as

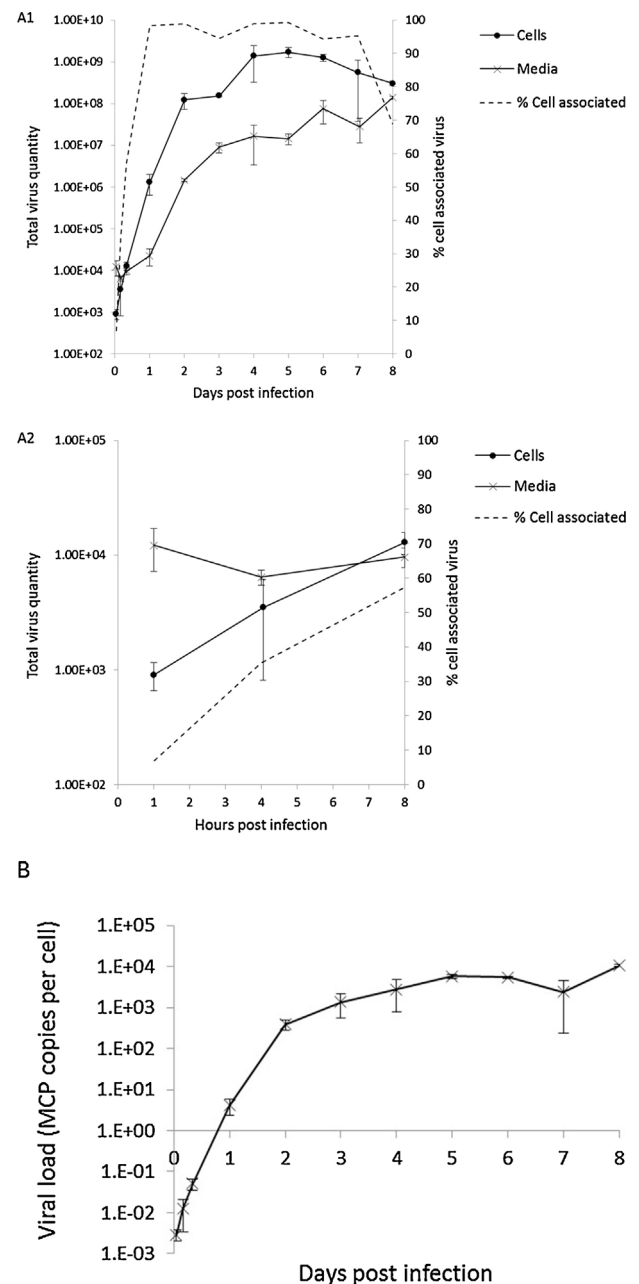


Fig. 3. Ranavirus growth in IgH-2 cells measured with the viral qPCR. (A) Plots show the viral quantities in the ‘cell’ and ‘media’ samples and the percentage of cell associated virus over (A1) the entire experimental period (eight days) and (A2) the first eight hours post infection. (B) Viral quantities were normalised by host cell quantity derived from the host qPCR to give viral loads. Data points are viral quantity means of duplicate cell culture wells for A1 and A2 and means of duplicate associated viral loads for B. Bars show the upper and lower range of the data.

observed in the current study and highlighted by others (Dimitrieva and Bucher, 2013). This complements work by Holopainen et al. (2011) by extending the range of the normalising host qPCR from a few fish species, to most vertebrates.

The host qPCR could also be applied to normalise infection burdens with other pathogens affecting other vertebrate hosts. This may prove convenient for lesser studied vertebrates where a paucity of sequence data complicates target selection and primer design. It also provides a useful tool for the normalisation of pathogens which infect multiple host species since the same normalisation qPCR can be applied. In the current study, the host qPCR was used to normalise the viral quantities in samples derived from host tissues and cultured cells but should be

equally applicable to swab samples. Swab derived DNA is prone to PCR inhibition so the host qPCR could act as a useful internal positive control in these instances (Hyatt et al., 2007; Kosch and Summers, 2013). Independent validation is recommended before applying the method to host species and pathogens not included in this study.

To demonstrate an application of the viral and host qPCRs, quantities of cell-free and cell-associated ranavirus were generated and the cell-associated viral quantities were normalised using the host qPCR to generate viral loads. More than 90% of the virus remained cell-associated throughout most of the infection cycle highlighting the need to incorporate steps to disrupt cell membranes during virus harvest. Gravell et al. (1968) demonstrated that the proportion of cell-associated frog virus 1 is also high. At 4 d p.i. the proportion of cell-associated frog virus 1 cultured in Chick Embryo and Fat Head Minnow cells was 99% and 70% respectively compared with 99% at the equivalent time point with an isolate of FV3 in IgH-2 cells in this study. Gravell et al. (1968) however, calculated this proportion from plaque-forming units (PFUs). Further study is required to determine the relationship between PFU and genome copies but if a relationship were to be established, the qPCRs described in this study could be a useful tool for the rapid approximation of PFU.

Molecular methods, including those described in this study are subject to a number of limitations. Firstly, they can inform on infection with ranavirus but cannot inform on disease status (Gray et al., 2009). Supportive tests such as histopathology must be used to resolve these two states (Gray et al., 2009; Rijks et al., 2016). Further, qPCR assays for ranavirus are likely to be subject to a degree of error in two ways. Firstly, replicated and unpackaged genomes and inactive virions will be amplified in addition to infectious virions (Holopainen et al., 2011). It is not known how total ranavirus genome copy number relates to the number of infectious virions. Secondly, ranavirus genomes are terminally redundant meaning that individual virions contain one whole genome plus an additional, repeated section (Goorha and Murti, 1982). This may introduce a degree of error when comparing viral loads since the proportion of the repeated section varies between *Ranavirus* species (Chinchar et al., 2009). In practical terms however, the degree of error introduced in this way can be considered very small in comparison to the orders of magnitude changes observed between ranavirus infected individuals.

In conclusion, while it is recommended that researchers always assess the suitability of analytical methods for their particular study, the qPCR method described here represents an effective tool for the sensitive and specific detection of AARVs, and the robust estimation of viral loads among diverse host species and tissues. The host qPCR will also be of use to those interested in determining infection burdens for other pathogens affecting vertebrate hosts and may have wider applications.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the

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